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METABOLISM AND EXCRETION OF ^{14}C-LABELLED DIETHYLCARBAMAZINE

BY

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(RECEIVED MARCH 24, 1955)

There has been no literature on the metabolism of piperazines since Hanzlik's paper in 1917 declaring piperazine to be valueless in gout. Hanzlik mentioned the general view that most of an oral dose of piperazine was rapidly excreted unchanged in the urine, but that some was oxidized in the body.

No further mention was made of piperazines in therapeutics until Hewitt and his co-workers (1947, 1948) investigated over one hundred piperazine derivatives for filaricidal activity on *Litomosoides carinii* infections in cotton rats. The outcome of this work was the introduction of diethylcarbamazine (1-diethylcarbamyl-4-methylpiperazine), which as a salt (Hetrazan, Banocide, Notezine) has been used extensively in the treatment of human filariasis.

While the mode of action of this drug was being investigated it was found that microfilariae are apparently unaffected when placed in a 1% solution at 37° C. for as long as 24 hr., yet if a dose of about 20 mg. base/kg. is injected into a cotton rat the number of circulating microfilariae is reduced by about 80% within 2–3 min. (Hawking, Sewell, and Thurston, 1950; Bangham, 1955). This contrast of an extremely rapid action *in vivo* with little or no apparent effect *in vitro* is unique among chemotherapeutic agents. As a first step in investigating it, the metabolism of this compound has been studied. The colour reaction described by Lubran (1950) has proved useful in studying the general behaviour of the drug in man; it is, however, not specific—there is a variable blank value for blood and urine estimations in untreated patients—and the technique does not estimate metabolites. Since there are no known sensitive specific and accurate chemical means of estimating piperazine compounds, material labelled with ^{14}C has been used in the experiments to be described.

Materials and Methods

Labelled Drug.—Diethylcarbamazine labelled with ^{14}C in the 3 and 5 positions of the piperazine ring was kindly synthesized by Dr. B. H. Chase and Mr. A. M. Downes (1953). A small amount of methylpiperazine similarly labelled was also available. Another batch of diethylcarbamazine kindly synthesized by Dr. H. R. V. Arnstein and labelled with ^{14}C in the methyl radical was used to study the metabolism of this radical. The specific activity of each of these batches of diethylcarbamazine was 1.5 μc./mg.

In neutral or alkaline solution diethylcarbamazine undergoes slow hydrolysis to methylpiperazine. A stock solution used for experimental purposes was kept at *p*H 6: this remained chromatographically pure for 4 months.

Doses.—All doses are expressed as mg. base/kg. body weight. The usual human oral dose is 2–4 mg./kg.; monkeys and rats were given doses varying from 2 to 25 mg./kg. as stated in each experiment. The radioactivity of each dose was adjusted so that each animal received 7–12 μc. as a single dose.

When given intravenously, the stock solution was neutralized with sodium bicarbonate immediately before injection, which was performed under light anaesthesia. Oral administration was by stomach tube.

Animals.—Male and female hooded rats (*Rattus*) and cotton rats (*Sigmodon*) weighing 150–250 g. were used. The male and female monkeys (*Macaca mulatta* and *Cercopithecus aethiops*) which were used weighed 3.8–6.5 kg.

Collection of Samples

Urine.—Urine from monkeys was collected at timed intervals by an indwelling catheter; a steady urine flow during the 12 hr. after intravenous doses was ensured by a concurrent slow intravenous saline drip. To facilitate more accurate collection of urine from rats the lower half of the body was depilated the day before use.

Expired CO_2.—For periods of observation of more than 30 min. rats were kept in a metabolism cage (capacity 1 l.). Air was sucked through this at 1 l./min. and then through a system of 3 bubbling tubes containing 10 N-NaOH connected in series with a

sentinel tube containing saturated barium hydroxide. For shorter periods of observation the rat was lightly anaesthetized with ether and its nose held in a small glass mask fitting round the neck. A steady swift suction was maintained through a tube from the mask to a glass spiral kept in liquid air. At the end of the observation period the liquid air spiral was isolated by taps and connected to the set of NaOH absorption tubes. The spiral was allowed to warm slowly and the released CO_2 was absorbed in the alkali. Carrier CO_2 was added by gently blowing through the spiral, so washing out the remaining expired CO_2. For short rapid successive collection-periods, two absorption spiral sets in parallel were used alternately. For monkeys a similar double absorption spiral set was used in conjunction with a mask fitting closely round the face.

The CO_2 was precipitated as $BaCO_3$ and washed with boiling distilled water, acetone, and ether. It was oven-dried, weighed, and an aliquot then plated and counted.

Estimation of Radioactivity

Samples were plated as infinitely thick preparations on 0.3, 1.0, 2.0, or 5 cm.^{2} discs. They were usually counted on an automatic sample changer under a thin-window GM tube. A perspex Harwell standard of 1 μc./g. giving approx. 1,000 c./min./cm.^{2} and a background (8–10 c./min.) were counted within at most 8 hr. of each sample. With the facilities that became available later they were counted alternately with each sample. All samples were counted for at least 1,000 counts. Samples giving less than twice the background were checked against the background count before and after.

By combustion of known amounts of labelled drug it was found that the $BaCO_3$ gave 11% more counts than calculated. This was assumed to be due to back-scatter by the heavy barium atom. Where possible, however, in calculating percentage recoveries the dose and the recovered sample were counted in the same chemical form, either as the organic compound or as $BaCO_3$.

Estimation of Total Radioactivity by Combustion.—(*a*) *In a liquid.* An accurate measured aliquot of the liquid (up to 0.1 ml.) was loaded on to a 2 cm.^{2} disc of Whatman No. 1 filter paper in a platinum combustion boat. This amount of paper gave 110–120 mg. $BaCO_3$, which is a convenient quantity for accurate weighing, easy handling, and plating. Smaller amounts of carrier were used if a low specific activity was expected. A simplified electrically driven and heated Pregl microanalytical line was used with a silica tube (11 mm. internal diameter) packed in the usual way. Dried CO_2-free oxygen was passed through the tube and the CO_2 produced by combustion was precipitated by bubbling through saturated barium hydroxide at 60–80° C. in sintered glass filter ampoules. Quantitative precipitation occurred, and the $BaCO_3$ could be washed, dried, and weighed in the same ampoule without atmospheric contamination. The specific activity of the known weight of $BaCO_3$ could then be used to estimate the radioactivity in the liquid sample taken. (*b*) *In a solid.* Weighed amounts of solid tissue which would give approximately 50–120 mg. $BaCO_3$ were combusted in the same way. Carrier carbon was added if necessary.

Accuracy of Combustion Method.—Calibration by combustion of known amounts of labelled crystalline substances showed a standard error of 3% for solid samples and 5% for liquid samples (including pipetting, weighing, and counting errors).

Separation of Metabolites

By Paper Chromatography.—Aliquots of urine or tissue homogenates were shaken thoroughly with 5–10 ml. 10 N-NaOH and 10–40 ml. chloroform. The chloroform was dried with sodium sulphate and concentrated by evaporation at room temperature. The concentrate was loaded on a strip of Whatman No. 1 chromatographic paper 2.5 cm. wide, and run as an ascending chromatogram using fresh distilled pyridine 350 parts, amyl alcohol 350 parts, and water 300 parts, as the mobile phase. A run lasting 16 hr. gave a solvent front at 30 cm. which provided adequate separation of spots. The chromatogram tank was kept in a thermostatically controlled room and the vapour inside maintained well saturated. Contamination with very small amounts of water or salt considerably altered the R_F values. Paper buffered at a high *p*H was found to give more reproducible R_F values.

Paper electrophoresis and numerous other chromatographic systems were tried, but none gave such good separation of spots.

All chromatograms were scanned for radioactivity by moving the strip automatically under a thin-window GM tube coupled to a ratemeter recording with a two-channel pen recorder.

All metabolites can be developed with iodine vapour, but neither this method nor the use of any other chromogenic reagents was specific or sufficiently sensitive for the minute amounts concerned.

Approximate R_F values were :

Piperazine base, 0.1 ; methylpiperazine hydrochloride, 0.1–0.2 ; unknown metabolite hydrochloride, 0.4 ; unknown metabolite base, 0.5 ; diethylcarbamylpiperazine, 0.60–0.65 ; diethylcarbamazine, 0.75 ; breakdown product of unknown metabolite, 0.8–0.9 ; methylpiperazine base, 0.8–0.9.

By Column Chromatography.—Zeocarb (200–400 mesh, 8–12% crosslinked) was prepared in the H form in the usual way. By successive decantation a grain size was obtained of such uniformity that there was no detectable layering after loading on the column.

On a pilot column of 80 g. resin (1.5 × 20 cm.) good separation of metabolites was achieved. Bases from the urine of a rat given 5 mg. of ring-labelled diethylcarbamazine intravenously were extracted into ether, which was then extracted with 5 ml. 1 N-HCl. (Although piperazine is not very soluble in ether, this solvent was used because of its low boiling point, since

some of the metabolites are both volatile and unstable as bases at higher temperatures.) The acid solution was loaded on the column and the bases eluted with 1 N-HCl. The eluate was tested for radioactivity at first by the conventional method of counting 0.1 ml. aliquots of fractions dried down on polythene discs, and later by passing it through a Helium-alcohol flow counter (Bangham, D. R., unpublished) which registered activity by means of a ratemeter coupled to a two-channel pen-recorder. This obviated much tedious sampling work and provided a continuous immediately available record of activity coming from the column.

Each peak of radioactivity was identified by paper chromatography and crystallization with carrier to constant activity. Methylpiperazine followed by diethylcarbamylpiperazine appeared as well-defined peaks; the unknown metabolite was well separated from the second peak, but was not quite separated from the fourth peak, diethylcarbamazine.

A large scale preparative column (600 g. Zeocarb, 2.8 × 85 cm.) was used with the intention of preparing some of the unknown metabolite for chemical identification. Five monkeys were injected intravenously with 50 mg. ($\equiv$14.1 μc.) ring-labelled diethylcarbamazine and their urine collected for the next 24 hr. in 10 ml. 1.0 N-HCl. The urine was freeze-dried in 12 bottles, 5 ml. 10 N-NaOH added to each bottle, and the metabolites extracted by shaking vigorously by hand with 6 × 100 ml. ether each. The ether was concentrated to 20 ml. under a 80 cm. fractionation column, and extracted with 10 × 2 ml. 0.5 N-HCl. This acid was loaded on the column, which was then eluted with 1.0 N-distilled HCl at a constant rate of 60 ml./hr. in fractions of 45 ml.

Fractions of each of the four peaks of radioactivity were pooled and run through a column of 600 g. Deacidite E in batches of 500 ml. The Deacidite had been prepared as recommended and washed with 2 l. warm methanol to remove colouring matter; it was regenerated between each cycle with 25 l. 2% $NaHCO_3$ and washed with 20 l. distilled water. The neutral pooled fractions were then made slightly acid again and freeze dried, before being made alkaline and extracted with 10 l. ether. This ether was finally concentrated under a 80 cm. fractionation column and the concentrate collected in a small weighed beaker.

The specific activities of these oily concentrates were less than calculated owing to contamination with colouring matter from the resins. The concentrates were therefore chromatographed on thick Whatman MMM paper in the pyridine-amyl alcohol-water system, and the specific peaks of radioactivity eluted with methanol.

Urine from a second group of six monkeys was dried similarly and the metabolites separated on a second column. Some improvement in the concentration of the last broad peak of radioactivity was achieved by changing the eluting acid to 2 N-HCl at the appearance of the first peak.

The treatment of the unknown metabolite is described below.

Estimation of Individual Metabolites by Carrier Techniques

Diethylcarbamazine.—Diethylcarbamazine hydrochloride and dihydrogen citrate were too hygroscopic when prepared for counting, since an automatic feed counter was used for radioactivity assay in which it was convenient to leave the specimen overnight.

Diethylcarbamazine gave a fine, stable non-hygroscopic white powder on precipitation with Kalignost $[(C_6H_5)_4B]^-Na^+$; Zeidler, 1952). One hundred milligrams carrier diethylcarbamazine (the dihydrogen citrate) was added to the solution—for example, urine—which was made strongly alkaline with sodium hydroxide and extracted with chloroform. This chloroform was filtered, concentrated, and extracted with 2 ml. 1.0 N-acetic acid. A filtered solution of Kalignost (150 mg./ml. water) was then added dropwise to the acetic acid extract until no more precipitation occurred. This precipitate was washed well with cold distilled water, dried in a vacuum desiccator, and plated for counting. The precipitate was recrystallized from acetone-petroleum (40–60°) to constant activity. Kalignost precipitates a wide range of organic bases, and several recrystallizations were necessary to produce constant activity in assays on samples of urine.

As an alternative method the chloroform was extracted with water and the drug precipitated with cold aqueous ammonium reineckate. Although this was much more specific for diethylcarbamazine, the conditions were difficult to reproduce and the salt tended to be unstable. The method was therefore discarded in favour of the one first described.

1-Diethylcarbamylpiperazine.—The urine sample was added to weighed amounts of carrier (80–100 mg. base) and the bases were extracted into chloroform as described for diethylcarbamazine. The chloroform was dried with sodium sulphate, filtered, and evaporated by warming gently. The residue was taken up in 2 ml. pure dry pyridine, about 500 mg. toluene-*p*-sulphonyl chloride was added, and the mixture heated to 100° C. for 15–20 min. After cooling it was poured into about 50 ml. cold distilled water. This gave a good yield of white crystals of the toluene-*p*-sulphonyl derivative, which could be recrystallized from hot ethanol-water. The method is relatively specific, and little reduction in count rate was observed after 2–4 crystallizations.

Attempts to form a dithiocarbamate with carbon disulphide were not regularly successful, and recrystallization sometimes gave yields too small for reliable counting. Theoretically the carbon disulphide precipitation method may be non-specific, and, in fact, after 6–7 recrystallizations with carrier, the count rate, although constant, was approximately three times that calculated from the toluene-*p*-sulphonate method. This contamination is so great as to suggest that the unknown metabolite may form a compound closely similar to the carrier.

Methylpiperazine.—Weighed amounts of carrier (about 150 mg. of the dihydrochloride monohydrate,

mol. wt. 191) were added to the urine sample. Extraction from acid solution, followed by extraction with chloroform from the alkaline solution, was carried out as above; the chloroform was filtered and alcoholic picric acid added dropwise. The crystalline dipicrate so formed (mol. wt. 558) was washed well with absolute ethanol and recrystallized to constant activity from dimethylformamide-absolute ethanol. About four recrystallizations were needed.

Piperazine.—Weighed amounts (about 200 mg.) of piperazine hexahydrate (mol. wt. 194) were added to the urine sample, which was made strongly alkaline with 10 N-sodium hydroxide and extracted with ether and then with chloroform. This chloroform was extracted thoroughly with 1.0 N-acetic acid and an aqueous solution of chromic acid added to the extract forming crystals of piperazine dichromate (mol. wt. 304). After standing for 30 min. in the cold these crystals were filtered off and washed with absolute ethanol. About five recrystallizations from hot water were needed to obtain constant activity.

RESULTS

Metabolism and Excretion of Ring-labelled Diethylcarbamazine

Table I shows the percentage recovery of an intravenous dose of diethylcarbamazine excreted in the urine in terms of total radioactivity (using ring-labelled material), of unchanged drug, and of

TABLE I

RECOVERY OF ^{14}C-LABELLED DIETHYLCARBAMAZINE FROM URINE

Animal	Position of ^{14}C in Drug Molecule	Dose (mg./kg.)	Route of Administration	% Excreted in the Urine in 24 Hr.		
				Total Radioactivity	As Diethylcarbamazine	As Methylpiperazine
Rat ..	Piperazine ring	24·0	i.v.	97%	18%	
,,	,,	27·0	,,	90%	20%	
Monkey	,,	2·8	,,	90%	11%	3·3%
,,	,,	2·9	,,	94%	12%	1·6%
,,	,,	15·0	,,	83%	18%	
,,	,,	7·4	Oral	70%	15%	
,,	,,	8·8	,,	78%	16%	
,,	Methyl radical	2·0	i.v.		11%	5·3%
,,	,,	2·0	,,		12%	3·5%
Rat ..	,,	20·0	,,	72%	10%	3·4%
,,	,,	20·0	,,	82%	14%	5·1%

methylpiperazine (using both ring- and methyl-labelled material). Fig. 1 shows cumulative curves of the percentage of total radioactivity and unchanged drug excreted in the urine (using ring-labelled material) and $^{14}CO_2$ in expired air (using methyl-labelled material, see below). In Fig. 2 the logarithm of the percentage of the administered dose excreted in 12 min. periods—based on the curves shown in Fig. 1—is plotted against a linear time scale. The log rate of excretion of both unchanged drug and expired $^{14}CO_2$ is approximately on the same straight line, which intercepts the abscissa at just over 3 hr. The slow excretion of $^{14}CO_2$ which occurs thereafter may be due to slow clearing of plasma bicarbonate and possibly some slow demethylation of another metabolite, but it is obvious that the major part of the expired $^{14}CO_2$ follows the excretion of unchanged drug. The slope of the log rate of excretion of total radioactivity has at least two components. During the first 3 hr. there is a rapid component,

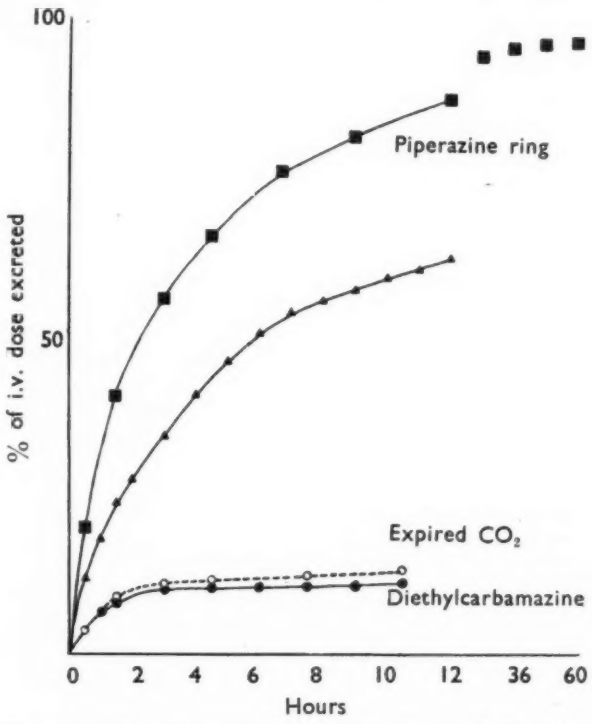

FIG. 1.—Graph showing cumulative excretion of: (1) ■ piperazine ring containing compounds in the urine; (2) ● unchanged diethylcarbamazine in the urine; (3) ○ expired $^{14}CO_2$; (4) ▲ the sum of (2) and (3) subtracted from (1). This is believed to represent the excretion of the unknown metabolite. Note change in time scale after 12 hr. Average of 4 rats given 20 mg./kg. diethylcarbamazine i.v. See Figs. 2 and 3 for further analysis of these curves.

which on extrapolation crosses the zero line at 6 hr. There is then a slow component from 3 hr. to approximately 10 hr. which on extrapolation crosses the zero at about 15 hr. If the sum of the percentage excreted as unchanged drug and $^{14}CO_2$ is subtracted from the total radioactivity excreted, the curve obtained is shown in Fig. 1 and the log rate of excretion of this is plotted in Fig. 2. This shows a slope with at least two components, an initial steep slope until approximately 2 hr. which would cross the base line at about 6 hr. and a

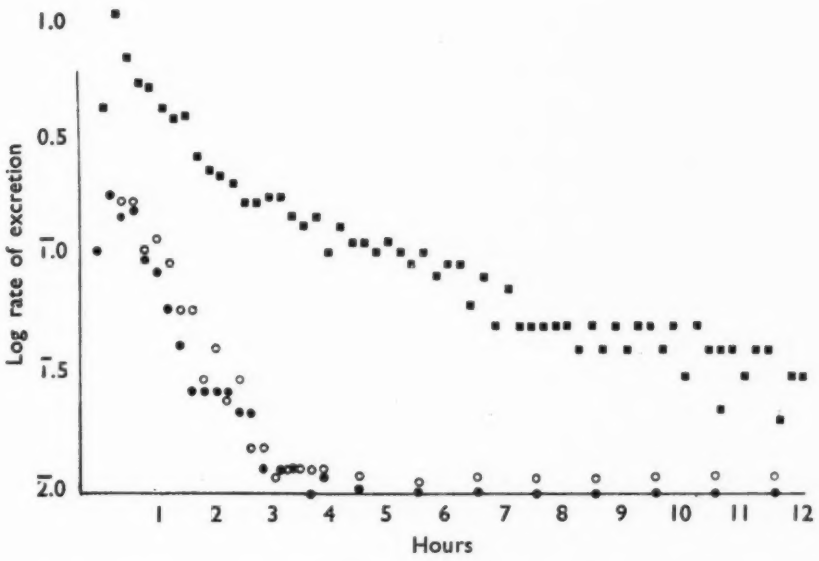

FIG. 2.—Graph showing the log of the percentage of the administered dose excreted in 12 min. periods, i.e. the log of the rate of excretion plotted from the curves shown in Fig. 1. The excretion rate of $^{14}CO_2$ (marked ○) closely follows the excretion rate of unchanged diethylcarbamazine (●) until 3 hr. after the dose.

second long component from 2 to 10 hr. which is believed to represent the rate of excretion of the unknown metabolite.

Total Excretion.—After intravenous administration of ring-labelled diethylcarbamazine dihydrogen citrate to either rats or monkeys, 80–100% of the injected radioactivity was found in the urine in 24 hr.—for example, two rats given 24 and 27.5 mg./kg. i.v. excreted 97 and 90% respectively; two monkeys given 7.7 and 15.0 mg./kg. i.v. excreted 100 and 83% respectively in 24 hr. After oral doses two monkeys given 7.4 and 8.8 mg./kg. excreted 70 and 78% respectively in 24 hr. Within the limits of accuracy of the methods used for collection of urine, combustion, and counting, the whole dose in terms of radioactivity appeared in the urine within 30 hr. Half the total activity injected was excreted in the urine in 3 hr. $\pm$ 20% both in rats and in monkeys. Samples of faeces which had been combusted contained negligible radioactivity.

Excretion as Diethylcarbamazine.—In rats and in monkeys 10–20% of an intravenous dose of diethylcarbamazine was excreted as unchanged drug in 24 hr. There was some suggestive evidence that after a larger dose more unchanged drug is excreted in the urine (Table I). In 4 rats given doses of 25–40 mg./kg. i.v. half of this amount was excreted in the urine in 50 $\pm$ 10 min. The maximum rate of excretion was 8.5%/hour during the period of 12–24 min. after the dose, which corresponds closely with the maximum rate of excretion (9%/hr.) of labelled methyl group as expiratory carbon dioxide during the same period (Figs. 1 and 2).

There was no evidence for retention of the drug, and after 9 hr. less than 1% of the dose was excreted as unchanged drug in the urine. After repeated injections more was excreted unchanged: a rat which had been given 24, 25, 23, 26 mg./kg. of methyl-labelled base (see below) intraperitoneally on four successive days excreted 7.4, 12, and 14% on the first, second, and fourth days while the drug was being administered, and only 0.5% on the fifth day. However, the $^{14}CO_2$ expired in the first 6 hr. after the first and last dose was 15.6% and 15.0% respectively of the previous dose given. This suggests that demethylation takes place to the same extent despite the increased excretion of unchanged drug.

Excretion as 1-diethylcarbamylpiperazine.—Monkeys and rats excreted between 5 and 15% of the dose as this substance. It was first detected in the urine about 20 min. after an intravenous dose.

Excretion as Methylpiperazine.—About 2–5% of the dose was usually excreted as methylpiperazine after intravenous doses. Under 1% was found in the urine as this substance in the first 9 hr. This probably represents a maximum, since diethylcarbamazine undergoes hydrolysis in alkaline solution, and urine was therefore maintained neutral or acid to prevent hydrolysis.

Excretion as Piperazine.—This is also excreted in small amounts; about 1–6% was identified as piperazine, and most of this appeared more than 6 hr. after the dose.

Excretion as the Unknown Metabolite.—As this has not been identified it cannot be directly estimated with carrier. Judged by the difference of the sum of the other metabolites and the total dose recovered it accounted for at least 60% of an intravenous or oral dose of 7–10 mg./kg. in the rat and of 2–30 mg./kg. in the monkey (Figs. 1 and 3). With lower doses it appeared to account for a larger proportion of the dose. This metabolite is discussed in more detail below.

Metabolism and Excretion of Methyl-labelled Diethylcarbamazine

Excretion as Expired $^{14}CO_2$.—Rats which had been given 13–15 mg./kg. of base intravenously expired 10–15% of the dose as $^{14}CO_2$. The time and rate of excretion in expired air closely followed that of the unchanged drug in the urine (Fig. 2). The slopes of the excretion curves were parallel until 3.2 hr. after the dose; thereafter a small, very slow release of $^{14}CO_2$ continued until 12 hr. after the dose. This might be accounted for by a slow demethylation of a metabolite taking place, together with the clearing of isotopically labelled plasma bicarbonate.

Rapidity of Demethylation and Excretion as $^{14}CO_2$.—If the action of a metabolite is invoked to explain the rapid biological effects of the drug, then there should be proof of the appearance of such a metabolite within the appropriate time. This cannot be done directly until the metabolite has been identified so that the carrier method of estimation can be used. Failing this, therefore, the excretion of the carbon of the methyl radical as expired $^{14}CO_2$ was studied, since it provides a simple if indirect indication of one stage of metabolism. The experiment is summarized in Table II. Four rats were given methyl-labelled drug intravenously and the excretion of $^{14}CO_2$ followed during the ensuing 0–2 and 2–5 min. periods. It can be seen from the results that demethylation is in fact rapid, if not extensive, during this time.

TABLE II

EVIDENCE FOR METABOLISM OF THE METHYL GROUP IN THE FIRST 5 MIN. AFTER AN INTRAVENOUS DOSE OF DIETHYLCARBAMAZINE LABELLED WITH ^{14}C IN THE METHYL RADICAL

	Dose (mg./kg. Base)	Radio-activity (μc. i.v.)	Period After Dose (min.)	% of Dose Expired as $^{14}CO_2$	Rate of Excretion (%/hr.)	Sample Counts Above Back-ground
Rat 1	24	7·6	0–5	0·128	1·54	57·5
„ 2	22	6·9	0–5	0·154	1·84	24·3
„ 3	18	7·0	0–2	0·0177	0·53	15·9
			2–5	0·050	1·0	30·7
„ 4	20	7·0	0–2	0·0178	0·53	12·0
			2–5	0·040	0·8	29·6

Rats 1 and 2 were given the drug without neutralization with sodium bicarbonate.
Rats 3 and 4 were given 10 mg. sodium bicarbonate with the dose.

Excretion in the Urine.—(*a*) *Total.* Two rats which had been given 20 mg./kg. i.v. excreted 72% and 82% of the total radioactivity in the urine in 24 hr. About 10–15% of this was unchanged drug and about 5% was methylpiperazine. (*b*) *As unknown metabolite.* Chromatography of urine extracts also showed a large amount of radioactivity at the same R_F as the unknown metabolite of ring-labelled drug. This was confirmed by running chromatograms of each of these extracts separately, and one loaded with some of each extract. Each of the three chromatograms showed a single large peak at the same R_F. It seems probable, therefore, that the methyl group is also present on the unknown metabolite. (*c*) *As*

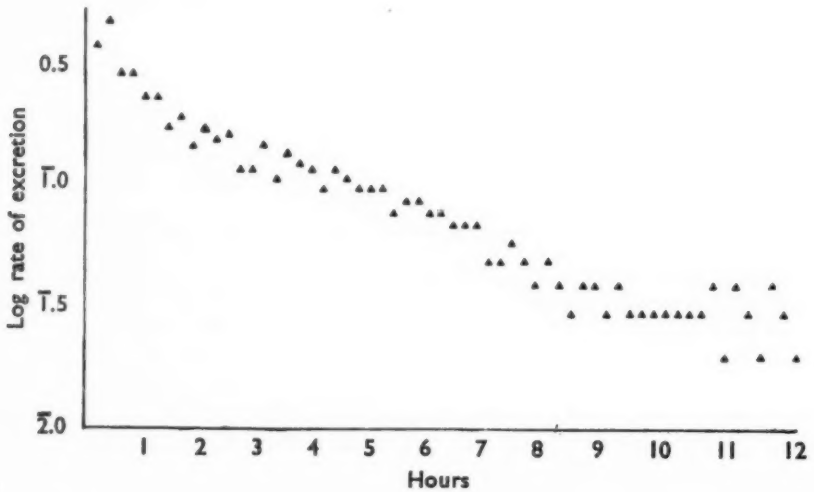

FIG. 3.—Graph showing the log of the excretion rate of the curve (marked ▲) in Fig. 1, which represents excretion of the unknown metabolite, plotted in the same way as in Fig. 2. A straight line could be drawn through the points from 2 hr. to 10 hr.

urine formate. The isolation of radioactive formate in the urine with the technique of Weinhouse and Friedmann (1952) also suggests that this methyl group carbon equilibrates with blood bicarbonate. Two rats which had been given 20 and 25 mg./kg. of drug i.v. excreted 0.3% in 4 hr., and 0.14% in 2 hr., and 5.17% in 2–4 hr. after the dose, respectively, as urine formate.

Entry of the Methyl Carbon into the Metabolic "Methyl Pool."—To see whether the methyl carbon also enters the metabolic pool the method of Mackenzie and du Vigneaud (1952) was used. They gave a rat 50 mg. choline hydrochloride by stomach tube daily for 8 days and again 50 mg. 30 min. before the test dose. In this way the methyl pool was kept saturated, and the carbon of any further methyl groups introduced (for example, i.v. administered drug) would be promptly excreted as respiratory $^{14}CO_2$. In their experiments using ^{14}C methionine as the test methyl group they found a 2–3-fold increase in CO_2 activity over 24 hr. and 10-fold increase in activity during the first hour.

No increase was found in two such experiments when rats were given 21.5 and 12.1 mg. base/kg. of methyl-labelled diethylcarbamazine.

Hydrolysis of Urine

After Ring-labelled Drug.—Two rats (200 g.) were given i.v. 20 mg./kg. of ring-labelled drug. Their urine was collected over 24 hr. in 2.0 ml. 1 N-HCl. Duplicate combustions of an aliquot showed that this urine contained 97 and 98% of the dose. Twenty millilitres of this urine were refluxed with 20 ml. 10 N-HCl for 10 hr. Duplicate analyses on this hydrolysate were carried out for piperazine and methylpiperazine, each being recrystallized and counted five times, as the dichromate and dipicrate respectively.

The results of two such hydrolyses showed 74.0 and 73.5% of the hydrolysate to be methylpiperazine and 22.3 and 20.6% to be piperazine. The remaining 5% may be accounted for by experimental error.

After Methyl-labelled Drug.—A similar hydrolysis of urine from another pair of rats treated with the same dose of methyl-labelled material showed that 80 and 86% of the dose was excreted in the urine, and 97% and 104% (experimental error = ± 5%) of the hydrolysate was methylpiperazine in two exactly similar experiments, each with duplicate analyses. This suggests that practically all of the ^{14}C in the urine is present as one or more substances which can be hydrolysed to methylpiperazine.

The Unknown Metabolite

This is the first metabolite to appear in the urine, and a substance with a similar R_F value appears in the blood within 2 min. after an intravenous dose. It has proved difficult to isolate because of its instability, and so far it has defied identification. Because of this it cannot be estimated directly by carrier, but judged by the difference between the sum of the other metabolites and the total amount excreted it seems to account for about 60% of the dose (Figs. 1 and 3, and see Discussion).

Isolation and Properties.—The attempt to isolate by column chromatography enough for chemical identification was disappointing on account of its instability. The few milligrams isolated by elution of the peak from the final thick paper chromatogram showed it to be an odourless, pale yellow, oily, basic material. This material is soluble in chloroform and ether but is more soluble in methanol. It is stable to acid (5 N-HCl for 5 hr. at 100° C. gave only partial hydrolysis), and to oxidizing agents (20 vol. hydrogen peroxide), and it can be boiled for 30 min. in dilute solution at *p*H 6 with little decomposition. It is, however, unstable in weakly alkaline solutions and is hydrolysed at *p*H 8 at 50° C. to a substance of R_F 0.8. The intermediate breakdown product (R_F 0.8) is less polar and more soluble in organic liquids (particularly methanol) than in water.

An attempt at a simple counter-current separation of these substances illustrates the instability of the unknown, and the relative non-polarity of its breakdown product. Shaking 29 mg. of the oily material obtained from the ether extract of the column-separated unknown—still contaminated with colouring matter from the resins Zeocarb 225 and Deacidite—in a 6 × 50 ml. mutually saturated butanol-distilled water system, converted two-thirds of it to the breakdown product, 90% of which stayed in the butanol phase.

At no time has more than 90% purity (radioactive) been achieved with the unknown substance since there is always some breakdown product with it. Further efforts at purification were made, but the yield from the column preparation was so small that it was not possible to characterize it chemically. There was, however, sufficient to obtain an infra-red spectrograph on the unknown substance as a base embedded in potassium bromide. The breakdown product crystallized from dimethylformamide as a few small amorphous polarizing crystals after 2 wk. at room temperature in a

desiccator. I am indebted to Dr. R. K. Callow for the following report on infra-red absorption spectra:

"Infra-red absorption spectra were measured in potassium bromide on a Perkin–Elmer Model 21 double-beam spectrometer with a rock-salt prism.

"Descriptions of infra-red absorption spectra for this group of compounds have not been traced in the literature. Piperazine, 1-methylpiperazine, 1 : 4-dimethylpiperazine, 1-ethoxycarbamylpiperazine, 1-diethylcarbamylpiperazine, and diethylcarbamazine were examined for comparison, all as liquid films. Simple relations between spectra and composition were difficult to find.

"The unknown metabolite and its breakdown product (as a smear) showed an absorption, with two peaks at 2,910 and 2,840 cm.$^{-1}$ (Ch stretching) forming a narrow band between 3,000 and 2,800 cm.$^{-1}$ This is characteristic of the unmethylated piperazine compounds examined. The N-methyl derivatives showed strong absorption extending to 2,700 cm.$^{-1}$ The unknown and its breakdown product (smear) also showed absorption at 1,725 cm.$^{-1}$ as a shoulder to a broad band at 1,650 cm.$^{-1}$ This suggests the presence of $-CO_2H$ or CO_2Et. 1-Ethoxycarbamylpiperazine is the only one of the examined substances which had absorption in this region. A band at 1,275 cm.$^{-1}$ in the spectrum of the unknown product gives some support to this suggestion. The change from the unknown to its breakdown product involves a change in the value of the maximum of the broad band between 1,700 and 1,600 cm^{-1} from 1,625 to 1,650 cm.$^{-1}$ No suggestion can be made as to the cause of this change.

"Crystals isolated from dimethylformamide were extracted with a little chloroform and the extract was evaporated on potassium bromide. The infra-red absorption of this material suggested that the material might be one component of the unknown, assuming the sample of the latter examined to be a mixture. There was a peak at 2,900 cm.$^{-1}$, a blunt peak at 1,660 (but no indication of a 1,725 band), and some absorption in the 1,450–1,350 region, including a peculiar spike at 1,385 cm.$^{-1}$ present in the unknown and doubtfully in its disintegration product.

"To sum up, although the infra-red spectrum of the preparation of the unknown metabolite suggests inhomogeneity, it may be consistent with the loss of the methyl group, and the possible presence of CO_2Et. Of the compounds tested it is most similar to that of 1-ethoxycarbonyl-4-methylpiperazine."

Other Possible Derivatives. — Various other possible compounds were added as carriers to urine containing the unknown substance: 1:4-dimethylpiperazine (R_F 0.4–0.5), diethylcarbamazine as a quaternary compound (methiodide crystallized as the picrate), 1-ethoxycarbonyl-4-methylpiperazine (as dihydrogen citrate), creatinine (as picrate), urea, the *N*-acetyl derivative of diethylcarbamylpiperazine (R_F 0.7–0.75), iminodiacetic and methyliminodiacetic acid, and 4-methyl-2:6-diketopiperazine were all tested, but no radioactivity was found on recrystallization. Furthermore, chromatograms of acid hydrolysates showed no ninhydrin spots characteristic of amino acids. It is concluded, therefore, that the unknown metabolite is not any of the above compounds, and is not attached to an amino acid.

Discussion

By the use of ^{14}C ring-labelled diethylcarbamazine it has been shown that the piperazine ring is almost entirely excreted in the urine in 24–30 hr. This is not surprising, since the piperazine ring does not normally occur in nature and it is unlikely that the body would break down or use such a ring structure. After intravenous administration 10–20% of the dose was excreted as unchanged drug, 8–15% as diethylcarbamylpiperazine, 2–5% as methylpiperazine, and 1–6% as piperazine. The remaining 60% was excreted as another unidentified metabolite. When methyl-labelled drug was given, about 10–15% of radioactivity appeared in expired carbon dioxide and 5% in the urine as formate. This suggests that the unknown metabolite must thus have retained its methyl group. It can be seen that the sum of the two demethylated compounds, diethylcarbamylpiperazine (10–15%) plus piperazine (1–6%), approximately equals the sum of $^{14}CO_2$ expired (10–15%) plus the amount excreted as formate (1–5%).

This is confirmed by the acid urine hydrolysis which yielded 20% as piperazine and 75% as methylpiperazine. Moreover, about 80% of activity appeared in the urine 24 hr. after injecting methyl-labelled drug, and this with the expired $^{14}CO_2$ amounts to the usual activity excreted 24 hr. after injecting ring-labelled drug.

The entry of methyl carbon into the metabolic pool depends very much on what molecule it comes from and on the dose of that substance. Whereas the methyl radicals of choline (Arnstein and Neuberger, 1953) and methionine (Mackenzie, Chandler, Keller, Rachele, Cross, and du Vigneaud, 1949) partake rapidly and extensively in the metabolic turnover of protein and other amino acids, it cannot be assumed that the methyl radicals of other molecules, e.g., 1-diethylcarbamyl-4-methylpiperazine, or *N*-methyl-labelled morphine (Elliott, Tolbert, Adler, and Anderson, 1954), necessarily take part readily in this methyl pool. The dose administered also considerably affects the amount expired as carbon dioxide. The excessive amounts of drug given in the above experiments probably saturated the methyl pool, and therefore excretion as expiratory $^{14}CO_2$ was at a maximum, so that

if any methyl group did enter into the protein and amino-acid cycle the amount would represent an insignificant percentage of the dose.

The expired carbon dioxide and urinary formate probably represent the total amount of demethylation, and together with the results of urine hydrolysis it suggests that the unknown metabolite retains the methyl radical. This suggestion is at variance with the results of the infra-red spectrum on the small amount of the unknown metabolite and its breakdown product (R_F 0.8). Both these have absorption peaks at 2,910 and 2,840 cm.$^{-1}$, which is characteristic of the unmethylated piperazine compounds so far examined, whereas the methylated piperazine compounds show strong absorption extending to 2,700 cm.$^{-1}$ Both the unknown substances show absorption spectra which are markedly different from that of methylpiperazine inasmuch as each has absorption peaks which the other has not.

Until the conditions for stability of the unknown metabolite are more clearly defined it may prove difficult to isolate enough for chemical characterization, particularly in view of the difficulty in crystallizing piperazine compounds. The method of isolation described proved wasteful on account of the extraction procedures necessary before and after separation on the column which involved exposure to a high *p*H and a certain length of time as the unstable free base, and the resin itself contaminated the concentrate with considerable amounts of colouring matter.

Summary

1. The metabolism of the antifilarial drug diethylcarbamazine has been studied using drug labelled with ^{14}C in the 3 and 5 positions in the piperazine ring. Separation of metabolites by paper and column chromatography, and their estimation by carrier methods, are described.

2. More than 95% of a dose of 2–25 mg./kg. given intravenously to rats or monkeys is excreted in 30 hr. in the urine as compounds containing an intact piperazine ring.

3. Of such an intravenous dose, 10–20% is excreted in the urine as unchanged drug, most of which is excreted in the first 3 hr. From 5 to 15% is demethylated and excreted in the urine as diethylcarbamylpiperazine. From 2 to 5% is excreted as methylpiperazine, and from 1 to 6% as piperazine; both these appear in the urine 9 hr. after the dose.

4. A fourth metabolite accounts for at least 60% of the dose. This substance has been isolated in crude form, but, because of its instability as a free base, has not yet been identified. (Infra-red spectrography of this substance suggests that it is similar to ethoxycarbonylpiperazine.)

5. Experiments with drug labelled with ^{14}C in the methyl radical indicate that 10–15% of the radioactivity of an intravenous dose (at the same dose levels) is excreted as $^{14}CO_2$, and 2–5% as urine formate. Radioactivity can be detected in expired carbon dioxide within 2 min. after an i.v. dose. Analysis of excretion curves of $^{14}CO_2$ and unchanged drug indicates that only the unchanged drug is demethylated to any great extent.

6. Hydrolysis of urine containing metabolites of drug labelled in the piperazine ring and in the methyl radical suggests that the unknown metabolite retains its methyl group.

It is a pleasure to thank Dr. A. Neuberger, Dr. G. Popjak, Dr. T. S. Work, and the many individuals who have given me advice. In particular I should like to thank Dr. J. Walker for all his help, Dr. H. R. V. Arnstein and Mr. A. M. Downes for much valuable help in the initial stages, and Mr. K. Hobbs for his unremitting technical assistance.

I wish to thank Brig. J. S. K. Boyd for the gift of the *N*-diethylcarbamylpiperazine.

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THE MODE OF ACTION OF DIETHYLCARBAMAZINE INVESTIGATED WITH ^{14}C-LABELLED DRUG

BY

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Investigation of the metabolism and excretion of isotopically labelled diethylcarbamazine (Bangham, 1955) showed that this drug is rapidly metabolized and excreted in the urine after intravenous or oral administration. Ten to 15% of the dose is excreted as unchanged drug, 10–15% as diethylcarbamylpiperazine, 2–5% as methylpiperazine, and 1–6% as piperazine. The remainder of the dose—approximately 60%—is excreted as a compound which has been isolated in crude form but not yet identified.

The present paper describes the distribution of the drug in the organs and tissues of the cotton rat (*Sigmodon*) and of the hooded rat (*Rattus*), the uptake of drug by microfilariae and adult worms, and the action of the drug on microfilariae in the first few minutes after intravenous injection.

METHODS

Estimation of Radioactivity

The methods for estimation of total radioactivity in solids and liquids, separation and estimation of individual metabolites, chromatography counting techniques and limits were as described by Bangham (1955).

Microfilaria Counts

The cotton rats which were used weighed 150–300 g. and were reared in this Institute and infected here with *Litomosoides carinii* (Hawking and Sewell, 1948). Only animals with heavy infections were used.

A blood sample was drawn into a calibrated 10 mm.^{3} siliconed dry pipette, diluted by taking up a similar volume of 1% potassium citrate, and discharged on to a grease-free slide marked with two parallel lines 2 cm. apart. The pipette was rinsed again with about 10 mm.^{3} potassium citrate which was spread with the sample over the area—2 cm. × slide width (2.5 cm.). The slides were allowed to dry flat before being stained. Great care was needed during staining to avoid lifting and detachment of the edge of the film. Slides were usually stained with Giemsa, since this method was less likely to detach fragments from the film than staining with haemalum.

The microfilariae were counted under a ×10 objective and ×10 eyepiece by examining the width of the film (2 cm.) at 5 constant distances apart, i.e., the same geometric areas on each different film.

Demonstration of the Rate of Disappearance of Circulating Microfilariae after Intravenous Administration of the Drug

Blood-sampling Methods.—In order to investigate closely the disappearance in the first few minutes, a technique for rapid repetitive and reproducible blood-sampling was needed. Initially samples were taken from the left external jugular vein at the root of the neck. A small hole was torn in the vein wall through which just enough blood for each sample was allowed to leak. The hole could easily be closed by firm pressure with a pledget of cotton wool and opened again by a light scratch.

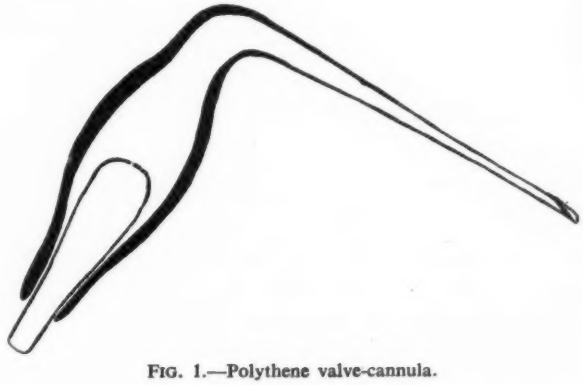

FIG. 1.—Polythene valve-cannula.

Later, a better technique for rapid sampling was used for experiments with radioactive drug. The technique (Bangham, A. D., personal communication) consisted in controlling the outflow from an artery by means of a small push valve in a polythene cannula.

One end of a small polythene tube was drawn down to fit closely round a short length of tapering glass rod. The polythene tube was cut short so that the glass projected 3–5 mm. when the valve was closed. If the projecting tip was pushed in, the valve was opened (Fig. 1). The other end of the tube was drawn out and cut obliquely for easy insertion into an artery. The cannula had a capacity of about 0.02 ml.

When the cannula was tied into the carotid artery, the arterial blood pressure forced the glass valve out

into the taper, and no blood could escape. The valve end of the cannula was then fixed into a base-plate carrying the rat so that the glass tip hung down vertically. A blood sample could then be taken by momentarily pushing the glass valve upwards.

Samples were taken on to perspex discs, 11 cm. in diameter, resting on three needle points set in a disc rotated by a 1 rev./min. Sangamo electric motor (Fig. 2). A second disc (hanging free of the needles and underneath the sample disc) was attached to the central movable core of an electromagnetic coil. On activation of the coil this second disc lifted the sample disc free of the needles. By adjusting the height of the coil core when raised, the sample disc could be made to impinge on the projecting glass valve of the cannula, so releasing a drop of blood. The size of the drop depended on the activation-time of the coil. A Palmer clock was used to control the timing and duration of the activation of the coil. With the cannula used, samples were released every 6 sec. and an activation time of about 1 sec. released samples measuring not more than 0.05 ml. blood.

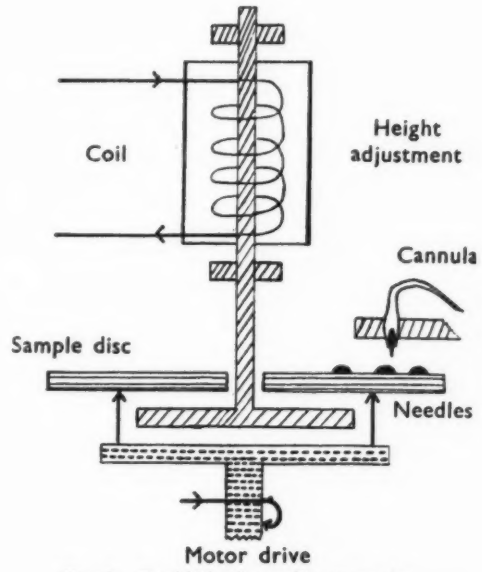

FIG. 2.—Rapid blood-sampling apparatus.

Procedure.—An infected cotton rat was anaesthetized with 1.5 ml. of 20% urethane intraperitoneally, and the cannula was tied into a carotid artery. Heparin (0.2 ml. of 1% soln.) was injected through the cannula, which was then fixed in the base plate. A syringe carrying the dose was connected in readiness to a fine needle in a superficial femoral vein. The base plate was adjusted over the sampling disc and 4 samples were taken at 10-sec. intervals to provide a base line for microfilaria counts. The dose of radioactive diethylcarbamazine (16.5 mg./kg. base in 0.25 ml. = 1.5 μc.) was injected during 10–18 sec. Just before the injection the sample timing was changed to once every 6 sec. and 10 samples were taken. The time was then readjusted to once every 10 sec. for 12 samples. Finally a sample was taken every 30 sec., the disc being rotated by hand. As each sample disc was loaded it was taken off and placed on ice, and another clean one was substituted.

The volume of blood removed 3 min. after injection of the dose was thus not more than 22 × 0.05 ml. = 1.1 ml. This was approximately 10–20% of the blood volume of a 200 g. rat. Over the first few minutes it provided a reproducible set of samples on which microfilaria counts, radioactivity assays, and chromatograms could be done.

For comparative purposes blood was assumed to have a constant carbon content, and counts on 1 cm.^{2} infinitely thick samples of barium carbonate are reported on this assumption. A chloroform extract of the blood samples was made directly they were taken from ice, and the extract was run as a chromatogram the same evening.

This technique differed from the blood sampling method first described in three ways : arterial blood was taken ; more samples were removed more rapidly ; and the animal had been injected with heparin. Heparin was necessary to prevent the slow clotting that occurred in the cannula despite heavy siliconing of the glass valve rod.

Uptake of Diethylcarbamazine by Tissues in the Rat in vivo

Rats of 150–200 g. weight were given an intravenous dose of piperazine ring-labelled drug and killed at various time intervals by a blow on the head. The organs were dissected and weighed after light blotting on filter paper. No correction was made for blood in the specimen, but care was taken to avoid any adipose tissue. The organ, or a piece of the tissue, was then combusted and the $BaCO_3$ counted. Rats were killed at 5 min., 10 min., 1 hr., and 6 hr. after an intravenous dose of approximately 25 mg./kg. base (7 μc./dose).

Uptake of Drug by Blood Cells in vitro

Fresh whole blood from normal hooded and cotton rats was heparinized (0.02 μg./ml.) and incubated at 37° C. with 5 or 50 μg. of labelled drug/ml. At 5, 15, 60, and 180 min. a sample was removed, and was centrifuged for 15 min. in a haematocrit tube ; measured volumes of plasma, red cells, and buffy coat were taken for assay of radioactivity by combustion. To obtain a greater number of white cells, two normal hooded rats were inoculated subcutaneously with 0.2 ml. of a yeast-saline suspension a week previously ; a sterile abscess developed at the injection site, and the white cell count of the blood was approximately twice the normal. No correction was made for plasma included among the cells, and they were not washed by resuspension because of the apparently easy diffusability of the drug.

No significant concentration by white or red cells was observed ; the drug seemed to diffuse evenly and rapidly among all cells.

Uptake of Drug by Worms of L. carinii in vitro

Adult *L. carinii* worms were collected from cotton rats killed with coal gas. Only undamaged lively worms were used after they had been separated into batches of males and females. They were incubated in 10 ml. of a 2/3 Ringer-glucose 1/3 horse serum medium containing 36, 58, or 65 μg. drug/ml. at 37° C. After 1 hr., when they were still wriggling vigorously, they were lifted out with a needle and rinsed for about 10 sec. in four amounts of 8 ml. of the drug-free medium, being dried carefully on filter paper between each rinse. After weighing they were either ground up with sand and carrier and the drug assayed as the reineckate, or they were dried in a vacuum desiccator for 24 hr., weighed, and combusted, and the activity assayed as $BaCO_3$.

Uptake by Microfilariae of Dirofilaria immitis

Microfilariae of this species were used in preference to those of *Litomosoides* because of their larger size and of the ready availability of blood from an infected dog. It is known that diethylcarbamazine is active against these microfilariae.

The dog was anaesthetized with intravenous pentobarbitone sodium and 24 ml. blood withdrawn into heparin. This blood was divided into four batches of 6 ml., each of which was injected into 75 ml. cold distilled water in a large conical centrifuge tube, and mixed by gentle shaking. Lysis of red cells was stopped after 5 min. by the addition of an appropriate volume of a concentrated saline solution to bring the salt concentration back to 0.9%. The microfilariae were then spun down gently for 10 min., and the supernatant saline was removed. The microfilariae were transferred with 10 ml. of Ringer-glucose solution into a smaller tube and centrifuged again to a total volume of 1.0 ml. To this was added 0.02 ml. of a stock solution of labelled drug to give a concentration of approximately 200 μg. base/ml. After 1 hr. in the incubator at 37° C. with occasional mixing, 9 ml. of Ringer-glucose was added and the solution centrifuged for 5 min. at 1,000 rev./min. Nine ml. of the supernatant fluid was replaced with fresh Ringer-glucose and, after mixing the solution, centrifuged again. After another similar wash, as much as possible of the supernatant fluid was removed and the sludge of microfilariae was transferred to a platinum combustion boat for weighing. The boat was then placed in a vacuum desiccator over P_2O_5 overnight and weighed again before combustion of its contents. Known amounts of carrier succinic acid were added to some small samples to provide adequate $BaCO_3$ for weighing and counting. No attempt was made to correct for washing solution included in the microfilarial sludge, but an assay was made on an aliquot of the last washing solution used (Table II). The three rinsings with Ringer-glucose eliminated a large amount of blood cell debris, and examination under the microscope showed little such material among the microfilariae.

Effect of Metabolites of Diethylcarbamazine on the Activity of Microfilariae in vitro

A suspension of microfilariae of *L. carinii* in 1/3 serum 2/3 saline was divided between two tubes, and test material to give a concentration of 1% was added to one tube. Both tubes were kept in a water bath at 37° C. and small amounts of the suspension were removed alternately from each tube and examined under the microscope every 2 min. An arbitrary scale of 4 points was used to indicate the activity of the microfilariae observed over 20 min.

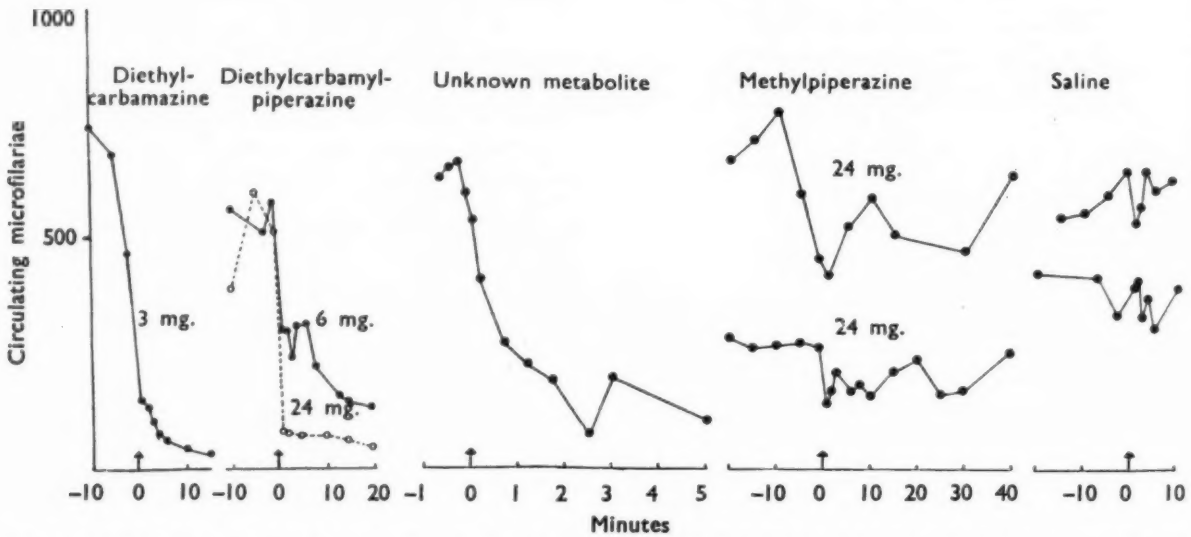

FIG. 3.—Effect of i.v. injection in 0.5 ml. saline of (1) diethylcarbamazine, (2) diethylcarbamylpiperazine, (3) unknown metabolite, (4) methylpiperazine, and (5) control injections of saline, on the number of circulating microfilariae in cotton rats (250 g.) infected with *Litomosoides carinii*.

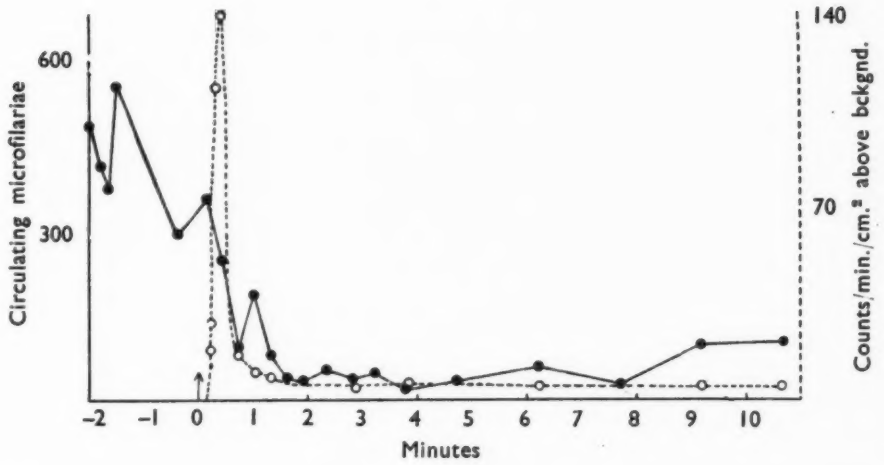

FIG. 4.—Correlation of drop in circulating microfilariae with the injection of ^{14}C-labelled diethylcarbamazine.

RESULTS

The degree of fluctuation in the number of circulating microfilariae of *L. carinii* that may normally occur is shown in Fig. 3 in the experiments where methylpiperazine or saline was injected. No immediate or sustained drop in numbers occurred after these injections. On the other hand, diethylcarbamazine caused the microfilariae to disappear rapidly from the circulating blood. Diethylcarbamylpiperazine exerted a similar effect when a higher dose was given. The result of one experiment, in which the breakdown product of the unknown metabolite was injected, showed that this substance was also active. Fig. 4 shows the fall in microfilariae in relation to the concentration of the circulating drug. Chromatograms of blood extracts suggested the presence of a substance with R_F 0.5, which is the same value as that of the unknown metabolite. Its identity cannot be established because a method of crystallization after isolation of the metabolite has not yet been found.

The average uptake of diethylcarbamazine by the tissues of the rat, determined 5, 10, 60, and 360 min. after an intravenous dose, was respectively 1.3, 0.77, 0.67, and 0.30% of the dose/g. tissue. There was no significant concentration of the drug by liver, common bile duct, muscle, kidney, spleen, lung, adrenal, skin, gut, vertebral column, testis, fat, or blood cells. None of these had an uptake of more than four times the average ; only the kidney maintained a higher average than the other tissues, presumably because of the presence of urine.

No significant concentration by leucocytes or erythrocytes of *Rattus* or *Sigmodon* was observed *in vitro* ; the drug seemed to diffuse evenly and rapidly into all cells.

The absorption of diethylcarbamazine by adult *L. carinii in vitro* is shown in Table I. Female worms varied markedly in size in different batches —presumably according to the age of the infection—so individual batches are reported. The difference in size may well account for the variation in the uptake of drug by male and female worms after 1 hr. The significance of the relative decrease in amount of drug found in female worms after 3 hr. is not known. When the results were expressed as μg./g. wet weight there was no significant concentration of drug by these worms above the level of the medium.

TABLE I
UPTAKE OF DIETHYLCARBAMAZINE BY ADULT WORMS OF *LITOMOSOIDES CARINII IN VITRO*

Worm		Wt. (mg.)		Incubation		Uptake of Drug by Filarial Worms	
Sex	No.	Wet	Dry	Time	Medium	μg./g. Wet	μg./g. Dry
					μg./ml.		
♀	61	100	14·8	1 hr.	36	60 } 55·5	401 } 300
♀		87	14·9	1 ,,	36	57	299
♀	57	17·5	4·5	1 ,,	36	21	81
♀		80·1	13·5	3 ,,	36	38	250
♀	100	98	16·5	1 ,,	58	28 } 35	175 } 216
♀		84·5	14·0	1 ,,	58	42	258
♂	54	16·4	4·8	1 ,,	58	19	64
♂		108·1	17·5	3 ,,	58	9	54
♀	69	228	37·6	1 ,,	65	59	355
♂		17	4·9	1 ,,	65	54	274

Table II shows the uptake of diethylcarbamazine by microfilariae of *D. immitis* incubated with the drug for 1 hr. *in vitro*. The concentration of drug in the organisms was only about one-third of that in the medium.

TABLE II

UPTAKE IN 1 HR. OF DIETHYLCARBAMAZINE BY MICROFILARIAE OF *DIROFILARIA IMMITIS IN VITRO*

Microfilariae		Medium	Uptake of Drug by Microfilariae		Concn. of Drug/ml. of Last Wash
Wet Wt.	Dry Wt.		μg./g. Wet	μg./g. Dry	
19·0 mg.	1·8 mg.	210 μg./ml.	66	595	1·0 μg./ml.
40·6 ,,	2·7 ,,	210 ,,	57	635	
69·3 ,,	6·2 ,,	200 ,,	64·6	712	1·65 ,,
—	1·3 ,,	200 ,,	—	654	— ,,

Neither diethylcarbamazine nor any of its four metabolites was found to have a significant effect upon the activity of *L. carinii* microfilariae *in vitro*.

DISCUSSION

The Possible Mode of Action of Diethylcarbamazine

It is well known that diethylcarbamazine rapidly reduces the number of circulating microfilariae of *W. bancrofti*, *W. malayi*, and *Loa loa*, and that those of *O. volvulus* die rapidly during treatment (Hawking, 1950; Hawking, Sewell, and Thurston, 1950). Its effect upon the adult worms of these species is still under dispute. Yet, so far, very little is known of the mode of action of this drug upon microfilariae, or that of the simpler polyvalent piperazine salts which are used for treating the larger helminths, *Ascaris*, whip worm, *Ancylostoma braziliensis*, etc. (Hawking, 1955).

It was soon established that diethylcarbamazine had little or no apparent effect upon microfilariae *in vitro* (Hawking *et al.*, 1950). These organisms can exist in a 1% solution of the drug in plasma, serum, or saline for many hours with no effect on their motility or histological structure. On injecting the drug intravenously in the cotton rat infected with *Litomosoides carinii*, however, the number of circulating microfilariae is reduced by about 80% in 2–3 min. Hawking *et al.* (1950) have demonstrated by histological methods that microfilariae are collected in the liver, which in the cotton rat contains most of the fixed reticuloendothelial system. Within a few hours the organisms are surrounded by phagocytes, and a few hours later they are obviously disintegrating.

This contrast of the rapidity of effect of diethylcarbamazine on microfilariae in the experimental animal, and *in vitro* respectively, is without parallel among chemotherapeutic agents.

There are three ways of interpreting how this drug exerts its effect. The drug may modify the surface of the microfilaria and act as an "opsonin"; it may be metabolized to form a substance, stable or unstable, which is directly lethal to the organism; or the drug may affect the host animal in such a way that it can "filter off" and phagocytose the organism.

The conception that the drug acts as an "opsonin" was put forward by Hawking (1950), and it is the only suggestion so far made in the literature. Several non-specific substances are known to act like opsonins for certain bacteria in biological systems *in vitro* (Topley and Wilson, 1946). Tannic acid, gallotannic acid, chrome alum and ferric salts, protamine, and globin all act in such a way. The metallic tanning agents and the strongly basic proteins, protamine and globin, are thought to combine with the carboxyl groups of bacterial proteins. These substances, like natural "opsonins," increase phagocytosis of bacteria *in vitro*, increase cohesiveness between particles, and lower the electric charge on the bacterial surface as determined by cell electrophoresis.

It would be difficult to demonstrate any one of these properties on microfilariae *in vitro*. Microfilariae normally exhibit a certain cohesiveness which does not apparently increase on exposure to the drug, while their large size (8×100 μ) and vigorous movement make it difficult to demonstrate phagocytosis outside the confines of a capillary. The interpretation of movement in an electrophoretic cell also becomes uncertain. This theory is therefore difficult to assess by these direct criteria. However, the experiments described above indicate that the clearance of microfilariae from the circulating blood by the drug is nearly as rapid as the clearance of foreign particles—such as dead bacteria or carbon black—introduced into the blood stream. Such a rapid clearing suggests a physical "filtering" by fixed macrophages or capillary endothelium. It is possible that within a minute of intravenous injection of drug the microfilariae, which are normally freely circulating, become denatured or modified so as to be recognized and treated as foreign particles. Microfilariae removed from the blood stream a few minutes after treatment with the drug still wriggle vigorously and look normal both before and after staining. They are not abnormally cohesive and they are not surrounded by phagocytes. Moreover, these few organisms which survive in the blood stream often do not disappear for some days despite repeated doses of drug.

Although little is known about the quantitative uptake of natural opsonins by bacteria, there is certainly no marked concentration of drug by microfilariae or adult worms (the females of which contain large numbers of immature microfilariae).

It would be of interest to see if this drug exerts any opsonizing effect upon bacteria in the biological systems already described in the literature.

The second possibility that a metabolite of diethylcarbamazine might be directly toxic to microfilariae seems unlikely when their rate of clearance from the blood stream is appreciated. Although the rapidity with which the labelled carbon of the methyl group appears as expired carbon dioxide suggests that metabolism is remarkably prompt (Bangham, 1955), the amount metabolized in the first two minutes is so small that any compound so formed would be present in about one-thousandth of the concentration of the drug administered. Moreover, chromatograms of blood and urine indicate that the unidentified metabolite is the first one to appear, and the evidence from hydrolysis experiments suggests that this metabolite still retains this methyl group. The simple demethylated compound diethylcarbamylpiperazine appears later in the urine and is known to be biologically less active.

It seems from the work of Hewitt and his co-workers (1947, 1948) that the piperazine ring is necessary for microfilaricidal activity, but that the alkyl radical itself is not essential in position 4. An increase in length of this radical results in diminished activity and increased toxicity. On the other hand, a carbethoxy group in position 1 with a number of different alkyl radicals in position 4 gave rise to a series of active compounds. The only compounds which lacked the carbethoxy group and which showed marked activity against microfilariae possessed an ethyl-, di*iso*propyl-, dimethyl-, or diethyl-carbamyl group in position 1. It is interesting, therefore, that the unknown metabolite showed an infra-red spectrum not unlike that of the 1-ethoxycarbonyl-4-methylpiperazine examined, and that this unknown metabolite is active *in vivo*. However, even if these other side chains were converted to a carbethoxy group, or if these side chains and the carbethoxy group were all metabolized to a common substance which was biologically active, the same anomalous situation arises, since neither this unknown metabolite nor any of the other metabolites have any apparent effect upon microfilariae *in vitro*. It is still possible to postulate an unstable intermediary metabolite being directly lethal to these organisms (this would be difficult to prove), but neither blood nor plasma incubated with drug, nor blood taken from a rat a few minutes after a dose, has any visible effect upon them.

Thirdly, it is possible that the drug has some effect upon the tissues of the host which enables it to remove microfilariae from the circulation. Several simple di-substituted piperazine compounds are in fact now recognized as having potent pharmacological properties (Roth, 1954; Jordan, Wheeler, Foye, and Orth, 1954). A number of such compounds examined by Roth (1954) showed a diversity of pressor blocking effects as well as antihistamine and local anaesthetic activity. At least two antihistamines used clinically (cyclizine hydrochloride (Norton, Colville, Light, Wnuck, Fanelli, and de Beer, 1954) and chlorcyclizine hydrochloride (Packman, Rossi, and Harrison, 1953)) contain a methylpiperazine group. The benzhydrylpiperazine group is also antihistaminic (Albro, Baltzly, and Phillips, 1949). Methylphenylpiperazine has been shown to possess marked adrenaline-blocking and reversing activity as well as local anaesthetic and other effects (Roth, 1954). Diethylcarbamazine itself, however, has few immediate pharmacological effects beyond producing a transient hyperpnoea, so it seems unlikely that any major circulatory change could account for the biological action. It is conceivable that diethylcarbamazine modified macrophages or the capillary endothelium in some way —possibly analogous to the action of the anionic detergent polyoxyethylene ethers on macrophages and tubercle bacilli (Mackaness, 1954)—so as to facilitate the trapping and phagocytosis of microfilariae. This also would be extremely difficult to prove. None of the tissues or organs of the rat examined showed any preferential uptake of the drug *in vivo* in the experiments described above.

The results of this investigation with isotopically labelled drug thus yield evidence that could support the theory that diethylcarbamazine has an opsonin-like action upon microfilariae, although direct proof of this mode of action must probably await a fuller understanding of the general anthelminthic properties of piperazines.

Summary

1. The clearance of microfilariae of *Litomosoides carinii* from the circulating blood by diethylcarbamazine and its metabolites in the first few minutes after an intravenous dose has been investigated. This clearance has been correlated with the presence of ^{14}C-labelled drug during that time.

2. Using labelled diethylcarbamazine, the uptake by organs and tissues in the rat has been examined at various intervals after an intravenous dose. No concentration above the average blood level was found constantly in any one organ or tissue.

The drug was not concentrated above the level in the incubating medium *in vitro* by rat red or white blood cells, or by adult worms of *Litomosoides carinii*, or microfilariae of *Dirofilaria immitis*.

3. None of the four metabolites of diethylcarbamazine—namely, diethylcarbamylpiperazine, methylpiperazine, piperazine, and the unidentified compound described in the previous paper—has any apparent effect upon the microfilariae of *Litomosoides carinii in vitro*.

4. The possible modes of action of this drug are discussed. The rapidity of clearance of microfilariae from the blood stream together with the other evidence described is consistent with the theory that the microfilariae are denatured or modified in some way so that they are treated as foreign particles.

It is a pleasure to thank Dr. F. Hawking for his continued interest and encouragement during the work in these two papers. I should also like to thank Miss W. A. F. Webber for supplying the infected cotton rats and Dr. A. D. Bangham for showing me the rapid sampling technique. I am most grateful to Mr. K. Hobbs for his patient and careful technical assistance.

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THE EFFECT OF COMPOUND 48/80 ON MAMMALIAN SKELETAL MUSCLE

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Compound 48/80 was introduced by Baltzly, de Beer, Buck, and Webb (1949) as a potent long-acting vasodepressor substance. Feldberg and Paton (1951) and Paton (1951) established that most of the symptoms occurring after the injection of 48/80 into animals can be attributed to histamine which is liberated by this drug in large amounts. On the other hand, Dews, Wnuck, Fanelli, Light, Tornaben, Norton, Ellis, and de Beer (1953) reported that although the response of the isolated frog gastrocnemius muscle to acetylcholine decreased in the presence of 48/80 they did not observe a similar inhibition after applying histamine. Furthermore, during their study of the restoration of the histamine-content of tissues Feldberg and Talesnik (1953) treated rats with increasing amounts of 48/80 until the histamine-store of these animals was largely depleted; these rats still exhibited some muscular weakness following an injection of 48/80 at a time when symptoms of an "anaphylactoid" reaction no longer occurred. Recently Gertner (1955) demonstrated a ganglion-blocking action of this drug.

The present series of experiments was designed to investigate the effect of compound 48/80 upon the function of mammalian skeletal muscle. Most of the *in vivo* experiments were done on rabbits, for their vascular system is relatively resistant to this drug (Dews *et al.*, 1953). Moreover, it was reported by Schachter (1953) that 48/80 is not very effective in releasing histamine in rabbits.

METHODS

Experiments in vivo.—Rabbits (2.0–3.5 kg. body weight) were anaesthetized with urethane (0.6 g./kg. i.v.+0.7 g./kg. i.p.) supplemented if necessary with small amounts of ether during the operation. The sciatic nerve was dissected in the thigh and crushed or severed. The calcaneal tendon was sectioned at its attachment to the heel and the movements of the foot—representing the contractions of the tibial muscle—were recorded on a kymograph. The jugular vein, trachea, and carotid artery were cannulated to permit intravenous injections, the application of artificial respiration, and the registration of the arterial blood pressure with a mercury manometer.

Rats of 200–300 g. body weight were anaesthetized with sodium pentobarbitone (50 mg./kg. i.p.). The right gastrocnemius muscle was freed from the surrounding muscles and the calcaneal tendon was severed and connected with the writing lever. The sciatic nerve was exposed and cut. A cannula was inserted into the left iliac artery pointing towards the bifurcation of the aorta. The pressure in the carotid artery was recorded by means of a double membrane tambour carrying a lever.

In both rabbits and rats the sciatic nerve was stimulated through paired electrodes with single maximal square-wave impulses of 3 or 5 msec. duration at a frequency of 12/min. For direct muscular stimulation a single electrode was fixed on the muscle, and a copper plate between the abdominal wall and the skin served as an indifferent electrode. Contractions were recorded isotonically.

Isolated Phrenic Nerve-Diaphragm Preparation.—This was prepared as described by Bülbring (1946); the rats weighed 120–160 g. The 50 ml. bath was filled with Tyrode solution containing 0.2% glucose and 0.1% $NaHCO_3$. Fresh, warm Tyrode solution was supplied from the bottom; excess fluid was removed by a bent glass tube connected with a suction pump. When the fluid had to be changed, the bath was rinsed first with about 200 ml. Tyrode solution and then (after 2, 4, and 6 min.) with about 100 ml. The temperature was kept at 37° C. Oxygen with 5% CO_2 was used to aerate the bath. A spring-loaded lever recorded the contractions. Maximal electric impulses of 1 or 2 msec. duration were applied to the nerve at a rate of 6 or 12/min.; pulses of 5 or 10 msec. were necessary for direct stimulation.

All drugs were dissolved in or diluted with 0.9% NaCl in water. Compound 48/80 was used as the base, histamine as the hydrochloride, physostigmine as the sulphate, neostigmine as the methyl sulphate, mepyramine, acetylcholine, and (+)-tubocurarine as the chloride. When histamine hydrochloride was tested on an isolated diaphragm an equivalent amount of NaOH was added to the Tyrode solution to prevent *p*H changes.

Results

Rabbits.—The contractions of the indirectly stimulated tibial muscle rapidly decreased after an intravenous injection of 48/80 (Fig. 1). A first dose of 3.3–5.0 mg. caused a depression of 50–95% which began almost immediately and was most profound 5–10 min. after the injection ; the muscle usually recovered in 30–70 min. A similar dose given after full recovery invariably had a much greater effect, sometimes causing complete paralysis for more than an hour, followed by a very slow recovery. After injection of 48/80 respiration became laborious, then shallow, and eventually stopped in 1–3 min.; apart from a slight transitory fall, the blood pressure was not altered by these doses.

Subsequently it was found that direct stimulation was still effective when indirect excitability was completely suppressed by 48/80 (Fig. 2). Furthermore, in animals completely paralysed by tubocurarine the contractions of the directly stimulated tibial muscle remained virtually constant for at least 10 min. after intravenous injection of 5, 10, or 20 mg. 48/80.

Histamine hydrochloride—up to 10 mg. intravenously—did not produce a paralysis like that after 48/80. Small amounts—up to about 0.5 mg. —had no visible influence. Larger doses—0.5 to 10 mg.—caused a severe drop in blood pressure and sometimes a depression of the tibial contractions ; but in contrast to the rather abrupt action of 48/80 the depression by massive amounts of histamine developed gradually. Indeed, in some experiments the maximal twitches gradually increased until the animal died (Fig. 3).

Although the intravenous injection of histamine did not produce an effect similar to that of 48/80, it was of interest to see whether antihistaminic drugs could prevent the action of the latter. This appeared not to be so, for after intravenous injection of 10 mg./kg. mepyramine hydrochloride 48/80 still paralysed the tibial muscle.

We next attempted to determine whether 48/80 acted by competition with acetylcholine or by depolarization. Some characteristics of the paralysis from 48/80 suggested a relation to that from tubocurarine—the inhibition of the single twitches was never preceded by a potentiation or by a spontaneous fasciculation ; the muscle did not maintain a tetanus with 40 pulses/sec. for 5 sec. applied to the sciatic nerve ; after such tetanizing stimulation the twitches were slightly larger than before (although this effect was not always so obvious as with tubocurarine) ; if tubocurarine was administered between two injections of 48/80, the second dose of 48/80 had a greater effect than the first. In many respects, therefore, 48/80 fulfils the requirements for true curariform action (Zaimis, 1954). Anticholinesterases, however, failed to antagonize the effect of 48/80. Physostigmine (1–2 mg.) or neostigmine (0.25–1.0 mg.), administered during partial or total paralysis caused by 48/80, did not significantly alleviate its effect. In control experiments identical doses of the two drugs readily relieved a comparable neuromuscular block by tubocurarine.

Rats.—In rats an intravascular injection of even small doses of 48/80 caused profound shock and so made it difficult to analyse the primary changes brought about by the drug itself in the muscle. Nevertheless, if rather large amounts were injected intra-arterially, an abrupt paralysis of the indirectly stimulated gastrocnemius muscle clearly preceded the fall of the arterial pressure (Fig. 4). In these experiments the impairment of muscular function obviously could not be secondary to circulatory failure ; it should be mentioned, however, that the paralysis did not always develop with equal rapidity. In a small series of six experiments, in which the doses varied from 1.0 to 3.0 mg., the degree of inhibition in the first minute ranged from 10% to 90%. These amounts were invariably lethal, death ensuing in 15–30 min.

Denervated Rat Muscle.—The right sciatic nerve of six rats was sectioned under ether anaesthesia and 10–13 days later we tested the sensitivity of the denervated gastrocnemius muscle to compound 48/80. The tone of the muscle did not change after intra-arterial injection of 0.1–6.0 mg. 48/80. If the muscle was stimulated directly when 48/80 was applied, the contractions remained constant for 90–120 sec., whereafter they decreased slowly —probably because of circulatory shock. As a control, acetylcholine (4–10 μg.) was injected repeatedly before 48/80 in three of the six rats ; the characteristic contracture of chronically denervated muscle was obtained.

Isolated Phrenic Nerve-Diaphragm Preparation. —Addition of 3–5 mg. 48/80 to the bath during regular stimulation of the phrenic nerve was followed by a gradual decrease of the contractions of the diaphragm. If the drug was washed out after a few minutes and a similar dose added when the muscle had recovered, the depression was more marked than at the first exposure. A dose that had scarcely any effect on a fresh diaphragm caused a rapid loss of contractility when applied for the third time (Fig. 5).

If 4 mg. or more of 48/80 was allowed to act upon a preparation for a prolonged period the

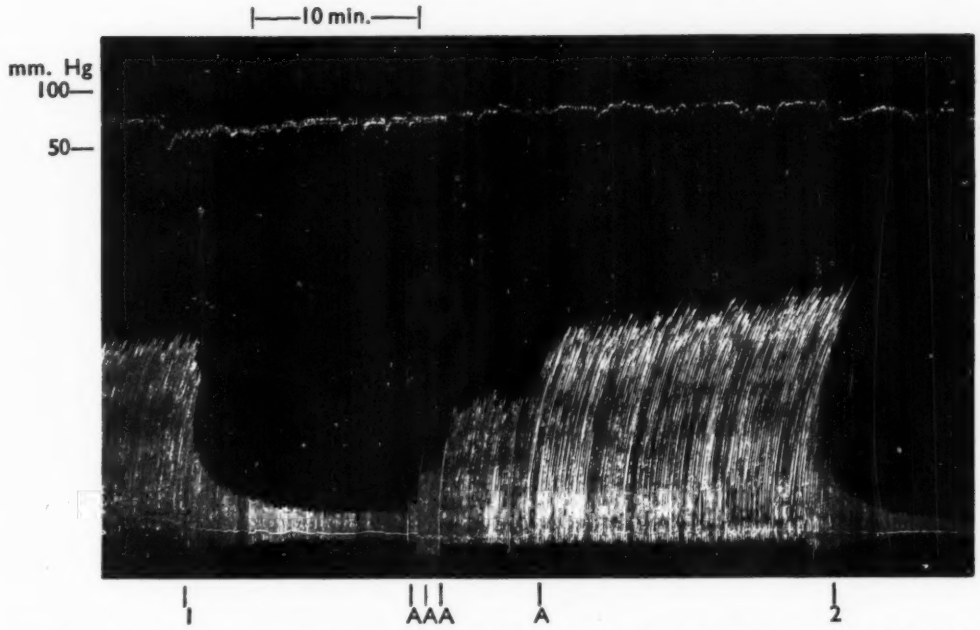

FIG. 1.—Rabbit, 2·2 kg. Upper tracing, pressure in the carotid artery; lower tracing, contractions of the tibial muscle. The sciatic nerve was stimulated with a frequency of 12/min. At 1 and 2 compound 48/80 was injected i.v. (5 and 2·5 mg., respectively). At A the kymograph was stopped. Total time between the two injections, 70 min.

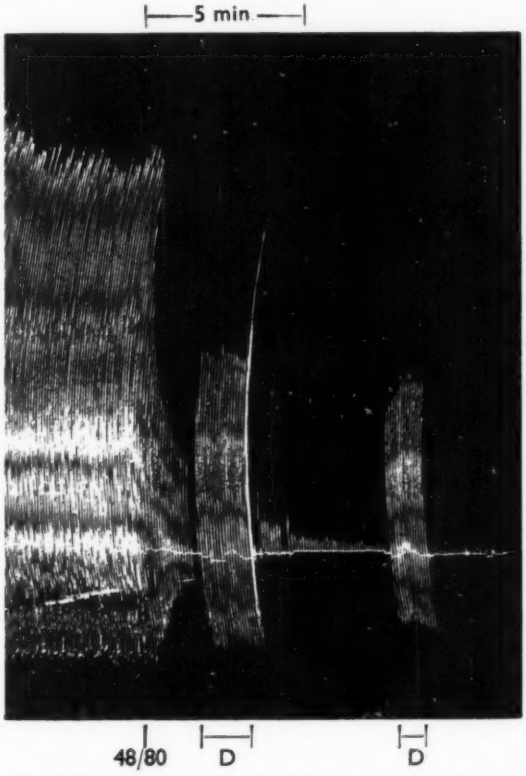

FIG. 2.—Rabbit, 2·1 kg. Record of the contractions of the tibial muscle. Intravenous injection of 3·5 mg. 48/80. (This animal had already been injected with 5 mg. about 80 min. earlier.) D, direct stimulation of the muscle.

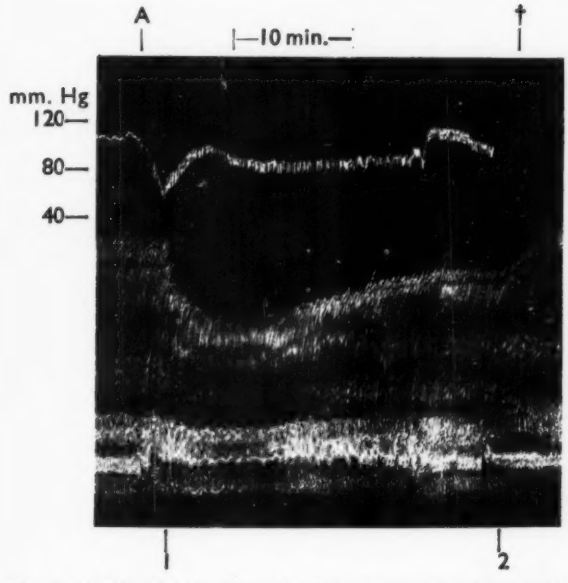

FIG. 3.—Rabbit, 3·3 kg. Records of blood pressure and contractions of the tibial muscle. 1, 4 mg. 48/80 i.v. 2, 0·5 mg. histamine-hydrochloride i.v. A, artificial respiration begins. †, death.

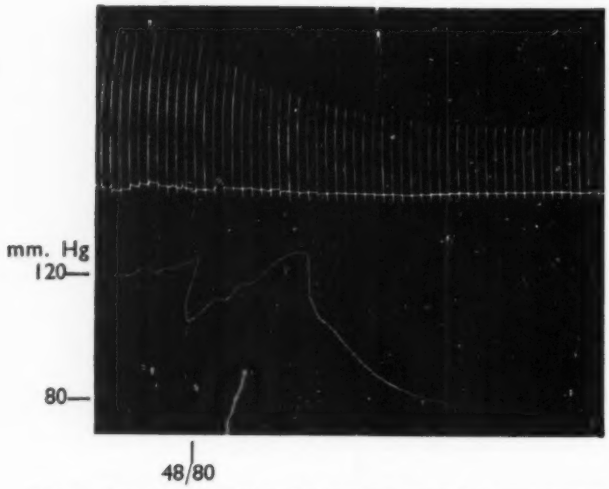

FIG. 4.—Rat 280 g. Upper tracing, contractions of the gastrocnemius muscle, frequency 12/min. Lower tracing, blood pressure. Intra-arterial injection of 3 mg. 48/80. The slight fall of the blood pressure at the moment of the injection was probably caused by traction on the iliac artery.

muscle eventually became refractory to nervous stimuli. The time necessary for such a complete inhibition varied from 3 to 30 min. and seemed to depend not only on the concentration of the drug but even more on the number of previous exposures. During a complete paralysis to indirect stimulation vigorous contractions could be obtained by direct stimulation, but, in contrast to the experiments on rabbits, these contractions tended to decrease if 48/80 was present in the bath for 5–15 min., especially when larger amounts (6–10 mg.) were used.

When 48/80 (4 mg.) was administered together with tubocurarine (40 μg.), the paralysis was more pronounced than with either drug alone.

Neostigmine neither prevented nor relieved the effect of 48/80. Fig. 6 illustrates the difference in the effect of neostigmine after tubocurarine and after 48/80. In three other experiments 2.5 μg. neostigmine was added 3 min. before 5 mg. 48/80, or 40 μg. neostigmine simultaneously with 4 mg. 48/80; after a further 3 min. the drugs were washed out and the muscle permitted to recover. Following this the same amount of 48/80 alone was added to the bath and kept there for 3 min.; then again the same dose of 48/80 together with neostigmine. In these tests too neostigmine failed to antagonize 48/80.

Histamine hydrochloride, in doses up to 20 mg. on three different preparations, did not have any effect upon the amplitude of the contractions of the diaphragm, stimulated by the phrenic nerve.

DISCUSSION

Our results seem to indicate that 48/80 in suitable doses causes a marked peripheral paralysis in rabbits and, under certain conditions, also in rats. The site of this action cannot be the muscle itself, because the contractions induced by direct stimulation are not affected to an appreciable extent at the concentrations used.

It may be assumed, for at least three reasons, that 48/80 itself and not liberated histamine is responsible for this effect: 1, administration of

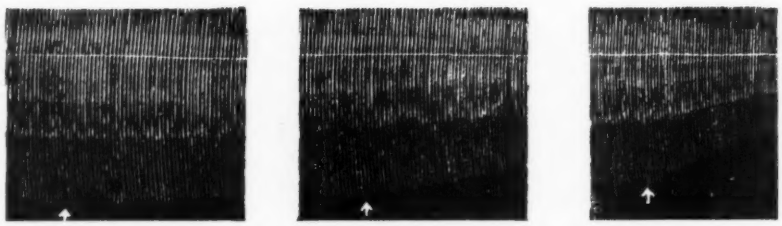

FIG. 5.—Isolated rat phrenic nerve-diaphragm preparation. Bath volume, 50 ml. Rate of stimulation, 12/min. Records taken from the same preparation. At each arrow administration of 2 mg. 48/80. Between the applications the drug has been washed out thoroughly.

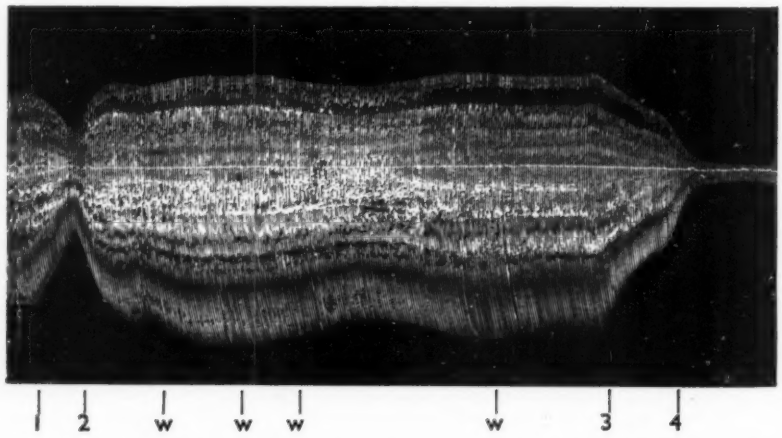

FIG. 6.—Isolated rat phrenic nerve-diaphragm preparation. Bath volume 50 ml.; frequency of stimulation 12/min. Contractions downward. 1, addition of 0·2 mg. tubocurarine to the bath. 2, 0·175 mg. neostigmine. 3, 5·0 mg. 48/80. 4, 0·175 mg. neostigmine. W, fresh Tyrode solution.

histamine failed to cause a similar paralysis in rabbits and isolated rat diaphragms; 2, if 48/80 was given repeatedly to the same rabbit or isolated rat diaphragm the depression became more profound with each subsequent administration, whereas the amount of histamine that is liberated diminishes with repeated exposure (Feldberg and Paton, 1951; Mongar and Schild, 1952); 3, high doses of mepyramine did not counteract the effect of 48/80 in rabbits. It is interesting in this respect that Wastl (1928) found fatigue of the artificially stimulated nerve-muscle preparation of a cat after the injection of histamine, an effect which she thought to be directly due to the drug. Eppinger, László and Schürmeyer (1928), who performed similar experiments on dogs, supposed that the decrease of the muscular contractions in histamine and peptone shock was due to decreased blood flow through the muscle.

Concerning the mechanism that is involved in the muscular paralysis caused by 48/80, no satisfactory explanation has yet been found. When tested in rabbits, some of its properties suggested a relation with tubocurarine; moreover, addition of the effects of 48/80 and tubocurarine was observed upon the isolated rat diaphragm. However, the inability of the cholinesterase inhibitors, physostigmine and neostigmine, to antagonize the paralysis by 48/80 is a strong argument against the view that the latter acts by competition with acetylcholine. On the other hand, the lack of stimulating effect upon denervated rat muscle makes it very improbable that 48/80 could have a depolarizing action.

A third known mechanism by which neuromuscular conduction can be blocked is inhibition of acetylcholine release at the motor nerve terminals. Gertner (1955) in his study of the effect of 48/80 on autonomic ganglia showed the release of acetylcholine to be unimpaired. It was therefore considered unlikely that the blocking effect of 48/80 on skeletal muscle would be due to inhibition of the release of acetylcholine at the motor endplate, although of course this drug might affect different tissues in different ways. Although our experiments did not exclude the possibility of axonal block by 48/80, we consider this rather improbable.

Most substances which block the conduction of impulses at the neuromuscular junction contain one or more quaternary or tertiary amino groups. An exception is mephenesin, which contains no nitrogen atom at all and yet exhibits some neuromuscular-blocking activity besides its action on the internuncial neurones (Berger, 1947). Compound 48/80 is a condensation product of *p*-methoxyphenylmethylamine with formaldehyde (Baltzly *et al.*, 1949). Being a secondary amine, it differs chemically from most other neuromuscular-blocking agents, and the question arises whether its action too is fundamentally different from the three main classes of peripheral paralysing drugs.

Summary

1. 48/80 causes a marked inhibition of the contractions of indirectly stimulated skeletal muscle in the rabbit. The direct excitability of the muscle is not affected.

2. Isolated rat diaphragms are depressed by 48/80 if stimulation is indirect, but not if it is direct.

3. Evidence is presented that the paralysis caused by 48/80 is due to the action of the drug itself and not to the liberation of histamine.

4. The results indicate that 48/80 does not act either by competition with acetylcholine or by depolarization at the motor endplate.

We are indebted to Professor B. Mendel for stimulating advice and criticism and to Dr. J. van Noordwijk for helpful discussions. We wish to thank Dr. W. Feldberg and Dr. E. J. de Beer for gifts of 48/80, and Mr. J. C. van Houten for technical assistance.

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THE ACTION OF ANALGESICS AND NALORPHINE ON THE COUGH REFLEX

BY

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Although the effects of various morphine-like analgesics on pain, respiration, body temperature, and gastrointestinal propulsion have been extensively examined and compared in several animal species, the antitussive action of these compounds has received relatively little attention. We therefore compared analgesics of several distinct chemical types, and also pholcodine and narcotine. For most of these comparisons we used the method of Domenjoz (1952) whereby respiratory efforts simulating those of coughs are elicited in anaesthetized cats by electrical stimulation of the superior laryngeal nerve. In addition, we examined the actions of codeine and of nalorphine on coughs due to various stimuli in cats, dogs, and guinea-pigs.

METHODS

Cats, dogs, and guinea-pigs were lightly anaesthetized with pentobarbitone sodium—30 to 40 mg./kg. i.p. and more as required i.v.—deep anaesthesia being avoided as this inhibited the cough reflex. In some experiments guinea-pigs were decerebrated under short ether anaesthesia.

Electrical stimulation of the central end of the cut superior laryngeal nerve drawn into a fluid electrode of the type described by Porter and Allamon (1936) was used to elicit respiratory efforts in cats. The stimulus consisted of pulses having a wave form approximating to that of capacitor discharge delivered at the rate of 6 to 10/sec. for periods of 3, 5, or 10 sec. from a blocking oscillator with constant voltage output (Dickinson, 1950). The pulse potential (usually 1–5 volts) was adjusted so as to cause a series of inspiratory gasps. These were recorded by a lightly sprung lever attached by a thread to the skin just below the sternum. An example of the type of tracing obtained is shown in Fig. 1. This method of stimulation was also tried in dogs, but, like Domenjoz (1952), we failed to produce a similar effect in this species.

A simple mechanical method—passing a thin polythene tube in and out of the trachea two or three times—was used to elicit short bursts of coughing in anaesthetized cats, dogs, and guinea-pigs.

Chemical stimulation was also used in unconscious animals. In cats and guinea-pigs 0.05 ml. (or less if effective) of SO_2 at atmospheric pressure, withdrawn from an SO_2 syphon in a syringe, was introduced into the tracheal cannula. This regularly caused coughing when, as in our experiments, an interval of about 8 min. was allowed between tests. In dogs these small volumes of undiluted SO_2 did not always induce coughing, and after larger amounts the animals frequently became refractory. More consistent responses were obtained by making the animal breathe an approximately 1 : 100 dilution of SO_2 in air from a bag until coughing occurred or for a maximum of 3 breaths, but some animals became refractory, and we have therefore reported only those experiments in which the SO_2 effect subsequently returned after suppression by an analgesic. Like the responses to electrical stimulation, those due to mechanical and chemical irritation were recorded from the abdomen except in three experiments in which a body plethysmograph, of the type described by Dawes, Mott, and Widdicombe (1951), was used to examine the nature of the responses more closely. These were found after each of the three types of stimulation to consist of one or more deep inspirations each followed by rapid forced expiration. The first inspiration was usually, but not invariably, preceded by a rapid expiratory movement.

Analgesics and other drugs were given intravenously in all experiments. In most experiments, including all of those in Table I, increasing doses in the series 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mg./kg. were given. Sufficient time for recovery, or 10 min. after ineffective amounts, was generally allowed between doses.

Development of acute tolerance to the respiratory depressant action of codeine was investigated in cats anaesthetized with pentobarbitone sodium by comparing the action on respiratory minute-volume (recorded as by Gaddum, 1941) of a single large dose of codeine with that of a series of increasing doses. The series was that used by May and Widdicombe (1954) : 0.5 + 0.5 + 1.0 + 2.0 + 4.0 + 8.0 + 16 mg./kg. given at intervals of approximately 5 min. The experiment was of cross-over design ; on the first day the series of doses was given to one cat and the single

large dose to the other cat, the cross-over test being carried out 3 days later.

The doses of the compounds are those of the salts referred to in Table I. Nalorphine was used as hydrochloride.

RESULTS

Antitussive Action

Cats.—The respiratory gasps caused by stimulating the superior laryngeal nerve were inhibited by each of the analgesics to an extent which varied with the dose. Table I shows the number of cats in a group, in which the inhibition was complete for at least 5 min. after progressively increasing doses of the analgesics. The relative potencies of the analgesics cannot be defined except at arbitrary levels, as the dose-probit response relationship is not the same for each compound. Thus, for example, the probit response increases more sharply with the dose of methadone than with the dose of morphine. Comparison of the ED50 values indicates that methadone is the most powerful suppressant of the reflex in these animals, its activity being perhaps slightly greater than that of piperidylamidone, about eight times that of morphine, twenty times that of pethidine, and about eighty times that of codeine.

TABLE I

THE NUMBER OF CATS IN WHICH THERE WAS COMPLETE SUPPRESSION, BY VARIOUS INTRAVENOUS DOSES OF ANALGESICS, OF COUGHS PRODUCED BY ELECTRICAL STIMULATION OF THE SUPERIOR LARYNGEAL NERVE

	No. of Cats Tested	Dose (mg./kg.) 0·03	0·1	0·3	1·0	3·0	10·0	30	Approx. ED50 (mg./kg.)
Morphine sulphate	7	0/3	1/7	3/7	5/7	7/7			0·4
Codeine (base) ..	6		0/6	0/6	1/6	2/6	6/6		4
Pethidine hydrochloride . ..	4		0/4	1/4	2/4	4/4			1
Methadone hydrochloride.. ..	6	1/4	6/6						0·05
Piperidylamidone* hydrochloride ..	4	1/4	2/4	4/4					0·1
Thiambutene† hydrochloride ..	2	0/2	0/2	2/2					0·2
Pholcodine (base)	4			0/4	1/4	3/4	3/4	4/4	2
Narcotine hydrochloride.. ..	2		0/2	0/2	0/2	0/2	0/2	1/2	30

* 6-Piperidino-4: 4-diphenyl-3-heptanone hydrochloride (Ofner, Walton, Green, and White, 1950).
† 3-Diethylamino-1: 1-di-2'-thienylbut-1-ene hydrochloride (Green, 1953).

Pholcodine, which is not a morphine-like analgesic, was slightly more active than codeine. Narcotine was relatively ineffective.

The duration of the suppression varied between compounds and with the dose administered. The effects of 3 mg./kg. morphine, 0.1 mg./kg. methadone, 3 mg./kg. pethidine, 0.3 mg./kg. piperidylamidone, and 30 mg./kg. pholcodine lasted for at least 2 hr., that of 3 mg./kg. codeine for about 1 hr., and those of 0.3 and 1 mg./kg. thiambutene for 15 min. and some 2 hr. respectively.

The effectiveness of codeine or narcotine was shown not to depend on the type of stimulus initiating the cough reflex. The effects of electrical, mechanical, or chemical stimuli were reduced equally by 0.3–1 mg./kg. and abolished by 3–10 mg./kg. codeine in each of three cats, and were unaffected by 10 mg./kg. and nearly abolished by 30 mg./kg. narcotine in one cat.

Dogs.—Coughs elicited by mechanical stimulation were abolished by 1 mg./kg. codeine in two of five dogs, and by 3 mg./kg. in each of five dogs. Coughs caused by SO_2 were abolished by 1 mg./kg. codeine in each of two dogs.

Guinea-pigs.—The dose of codeine required to suppress respiratory efforts due to either mechanical stimulation or SO_2 was approximately 10 mg./kg. in one decerebrate and two anaesthetized guinea-pigs.

Narcotine was ineffective at 10 mg./kg., and even 30 mg./kg. abolished the effects of mechanical and chemical stimuli in only one of two guinea-pigs.

Antagonism by Nalorphine

Nalorphine, in doses varying between 3 and 30 mg./kg., did not abolish the cough reflex induced by electrical, mechanical, or chemical stimulation in three anaesthetized cats; it did not abolish the reflex to mechanical stimulation in five unanaesthetized dogs; and it was likewise ineffective against mechanical or chemical stimulation in four anaesthetized and two decerebrate guinea-pigs.

Nalorphine rapidly abolished the antitussive action of morphine-like analgesics. The return of electrically induced respiratory efforts which had been suppressed by morphine, after the intravenous injection of nalorphine, is illustrated by Fig. 1. The effective dose of nalorphine varied with the dose and potency of the analgesic, and was of a similar order of magnitude to that antagonizing other morphine-like effects, (e.g., respiratory depression in these experiments). The following results obtained from a small number of tests provide a very rough estimate of the effectiveness of nalorphine against various doses of the analgesics. A dose of 0.1 mg./kg. nalorphine abolished the action of 0.3–1 mg./kg. morphine, of 0.1 mg./kg. methadone, and of 0.3 mg./kg. piperidylamidone; 0.3 mg./kg. nalorphine abolished the action of 1–3 mg./kg. morphine, of 1 mg./kg. thiambutene, and of 3 mg./kg. codeine; 1 mg./kg. nalorphine abolished the action of 0.3 mg./kg. methadone and of 3 mg./kg. pethidine; and 3 mg./kg. nalorphine abolished the action of 1 mg./kg.

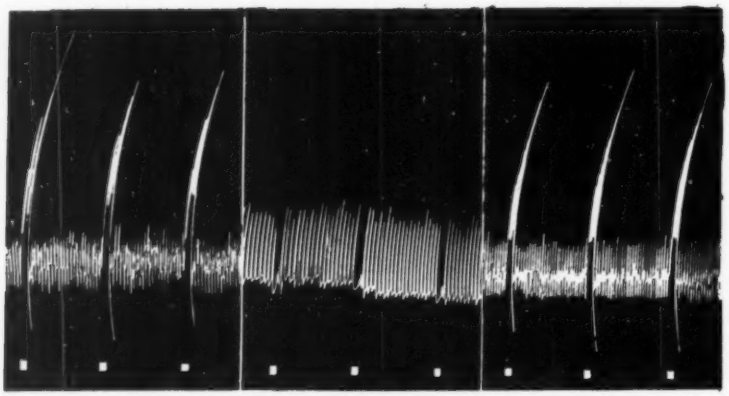

FIG. 1.—Record of coughs caused by electrical stimulation of the superior laryngeal nerve in an anaesthetized cat. Stimulus: 6 cycles/sec. for 10 sec. every 2 min. A, Initial. B, After 1 mg./kg. morphine sulphate i.v. C, After 0.3 mg./kg. nalorphine hydrochloride i.v. given 10 min. after the morphine. Inspiration is represented by an upward movement.

methadone and of 10 mg./kg. codeine. Nalorphine was equally effective in reducing or abolishing the suppressant action of codeine on coughs due to electrical, mechanical, or chemical stimulation in each of three cats. *N*-propylnormorphine (10 mg./kg.; lower doses were not tried) also eliminated the antitussive action of codeine (20 mg./kg.) in a cat. In an anaesthetized dog the suppressant action of 10 mg./kg. codeine on mechanically induced coughs was abolished by 1 mg./kg. nalorphine. Likewise in one decerebrate and two anaesthetized guinea-pigs, 1–3 mg./kg. nalorphine prevented or abolished the action of 10 mg./kg. codeine on mechanically or chemically induced coughs.

Some observations made on a dog with a natural cough, readily initiated by exposure to cold, are of interest. The cough was completely suppressed by 3 mg./kg. codeine i.v., but returned soon after giving 1 mg./kg. nalorphine i.v. Nalorphine, 1 mg./kg., alone failed to reduce the cough when tested on the following day; 3 mg./kg. did not abolish the cough but may have been responsible for a slight reduction in its severity. In another dog, natural coughing suppressed by methadone was restored by *N*-propylnormorphine.

In anaesthetized cats the antitussive action of 3 mg./kg. pholcodine or 30 mg./kg. narcotine was not antagonized by 10 mg./kg. nalorphine.

Effects on Breathing

Morphine, codeine, methadone, piperidylamidone, and thiambutene frequently reduced the rate of breathing in anaesthetized cats at doses the same as, or slightly above, those suppressing the respiratory response to stimulating the superior laryngeal nerve (Table II). With doses which just suppressed this response, the percentage reduction of respiratory frequency was usually small, e.g., a mean reduction of 21% (S.D. $\pm$9) with 0.1 mg./kg. methadone which abolished the cough in each of six cats, but in two cats codeine caused a great, though temporary, respiratory depression, and in two cats morphine caused persistent reduction (50 and 90%). Pethidine suppressed the cough without depressing respiration. Pholcodine depressed the respiration in one cat, and increased the frequency in others. Narcotine (10–30 mg./kg.) caused brief apnoea followed by an increase in frequency of breathing.

Antitussive doses of codeine (1–3 mg./kg.) had no effect on respiration in three anaesthetized dogs, and slightly increased the rate in two others; 10 mg./kg. codeine had no effect in one guinea-pig and reduced the frequency by about 30% in another.

Codeine caused rapid breathing in one of six cats in our experiments, and three of six cats in those of May and Widdicombe (1954). Further investigation showed that the action of codeine depended on the dose schedule. Thus, in a cross-over test using two cats, single large doses (8 mg./kg.) greatly decreased the respiratory minute-volume, whereas the same amount or more in divided doses produced rapid shallow breathing (Fig. 2). In other tests, although first doses of 1 or 3 mg./kg. codeine caused marked respiratory depression, later doses of 3 to 10 mg./kg. caused less depression. The development of tolerance to the depressant action of codeine may have been more marked in the experiments of May and Widdicombe (1954)—who gave the series of doses in Fig. 2 at 10-min. intervals—than in ours with larger dose steps and a greater interval between

TABLE II

THE NUMBER OF CATS IN WHICH THE FREQUENCY OF BREATHING WAS REDUCED BY AT LEAST 30%, 2 MIN. AFTER VARIOUS INTRAVENOUS DOSES OF ANALGESICS

	No. of Cats Tested	Dose (mg./kg.)				
		0·1	0·3	1·0	3·0	10·0
Morphine	7	0/7	0/7	2/6	2/2	
Codeine	6	0/6	0/6	1/6	3/6	3/5
Pethidine	4	0/4	0/4	0/4	0/2	0/1
Methadone	6	1/6	1/2			
Piperidylamidone ..	4	0/4	1/2			
Thiambutene	2	0/2	1/2	1/2		
Pholcodine	4		0/4	1/4	1/4	0/1

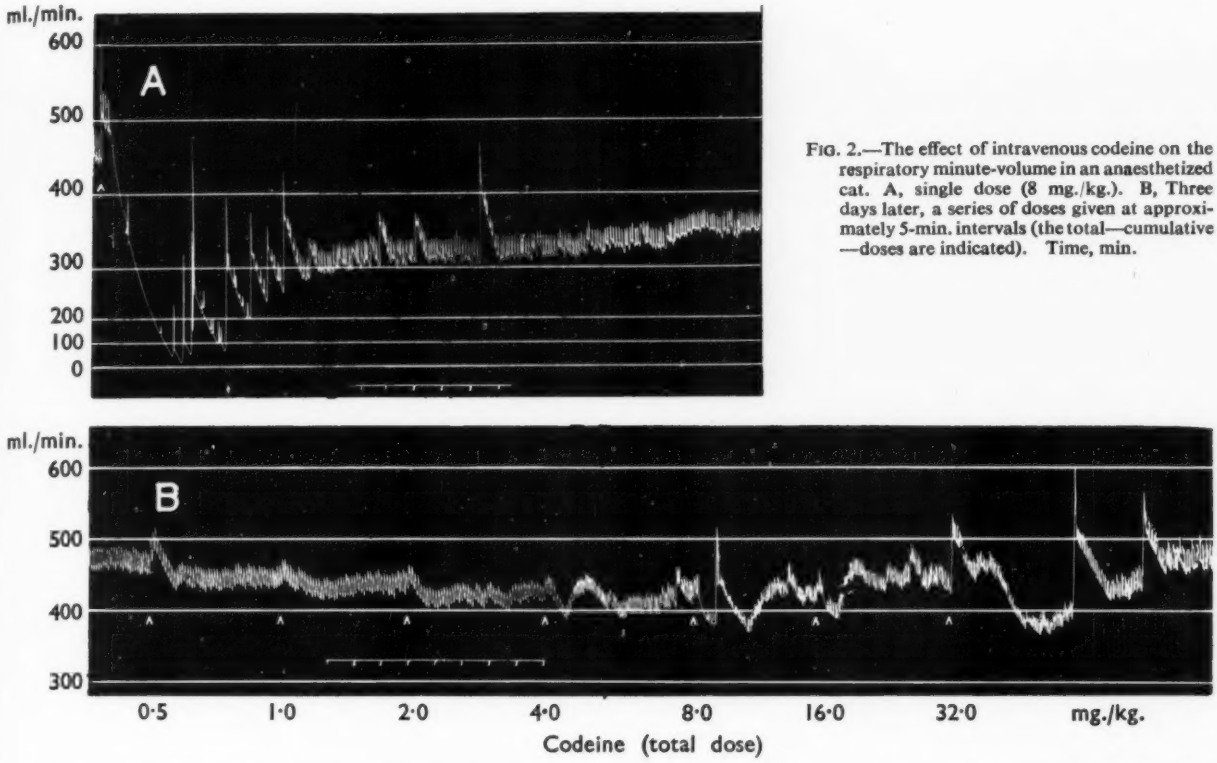

FIG. 2.—The effect of intravenous codeine on the respiratory minute-volume in an anaesthetized cat. A, single dose (8 mg./kg.). B, Three days later, a series of doses given at approximately 5-min. intervals (the total—cumulative—doses are indicated). Time, min.

doses. Respiratory depression was predominant in the experiments of Toner and Macko (1952), who gave single doses of 1 mg./kg. There was no indication in our experiments of acute tolerance developing to the antitussive action of codeine or other analgesics.

The dose of nalorphine that eliminated the respiratory depressant action of the analgesics was usually about half that which eliminated the antitussive action. This is in keeping with the observation that analgesics usually suppressed the cough reflex before they depressed the respiration.

DISCUSSION

In all our experiments the respiratory gasps, caused by electrical stimulation of the central end of the superior laryngeal nerve in the cat, closely resembled those caused by mechanical or chemical irritation of the bronchial system, and they responded in the same way to analgesics, to nalorphine and to narcotine. This indicates that the main effect observed during stimulation of this mixed nerve arises from excitation of afferent fibres which are the same as, or similar to, those excited by mechanical or chemical stimuli. This effect may therefore justifiably be used for the investigation of antitussive action.

The doses of the various compounds which suppressed the response to electrical stimulation of the superior laryngeal nerve in anaesthetized cats (Table I) are—allowing for minor variations in technique, and the considerable variations in the sensitivity of different cats to morphine and codeine—in keeping with the results obtained for morphine, codeine, and methadone by Domenjoz (1952) and for codeine by Toner and Macko (1952), who used similar methods. They are also of the same order of magnitude as the doses of morphine, codeine, and pholcodine which May and Widdicombe (1954) found to suppress coughs—referred to, in their paper, as inspiratory efforts—caused by mechanical or chemical irritation in anaesthetized cats. Kasé (1952, 1955) found that, for suppression of coughs caused by intense mechanical stimulation of the trachea in conscious dogs, the minimum effective intravenous doses were approximately as follows: morphine, 0.5 mg./kg.; codeine, 3 mg./kg.; pethidine, 3 mg./kg.; methadone, 0.5 mg./kg.; and narcotine, 10 mg./kg. With the exception of the methadone dose, which is somewhat greater, these amounts are similar to those found by various methods to be effective in cats. Further, we have found, using mechanical or chemical irritation, that the effective dose of codeine is similar in cats, dogs and guinea-pigs, and that the effective dose of narcotine was similar in cats and guinea-pigs. Therefore in these experiments the activities of compounds in suppressing

the cough reflex have not varied to any great extent either with the stimulus used for initiating the reflex or with the species. Further, effective doses in lightly anaesthetized animals have been similar to those in conscious animals. The agreement between the results obtained in such experiments is, however, not complete, for in a preliminary report Konzett and Rothlin (1954) state that 1 mg./kg. narcotine suppressed coughs caused by mechanical stimulation in anaesthetized cats, whereas we and Kasé (1955) find the minimal effective dose of this drug to be at least 10 mg./kg.

Nalorphine itself did not abolish the cough reflex, but abolished the action of morphine-like analgesics in all the experiments described. *N*-propylnormorphine, which shares other actions of nalorphine (Green, Ruffell, and Walton, 1954), showed the same antagonism to the suppressant action of codeine. The effective doses of the analgesics and nalorphine in these experiments are of the same order of magnitude as those known to produce effects on pain threshold, respiration, body temperature, and gastrointestinal propulsion. It is therefore concluded that similar drug receptor sites and mechanisms may be involved in the causation of these effects.

In contrast with the above findings, Winter and Flataker (1954) report that the frequency of coughing in conscious dogs and guinea-pigs forced to breathe mild irritants is reduced by low subcutaneous and oral doses of nalorphine, narcotine, or codeine, and that the effect of nalorphine is synergistic with that of codeine and narcotine. The effective doses determined by Winter and Flataker (1954, 1955) are, as we and others have shown, much less than those needed to depress or abolish the cough reflex or other nocioceptive reflexes. The effects they observed are therefore probably caused by some other mechanism, such as a decreased perception of the mild irritant or a depressed psychological reaction to it, for coughing during exposure to mild irritants is to some extent voluntary. Further, the reduction by small doses of narcotine of the frequency of coughing during anaphylactic shock in guinea-pigs (Winter and Flataker, 1955) cannot easily be explained by suppression of the cough reflex. The question arises: which type of experimental method is the most suitable for selecting new antitussive drugs for clinical investigation ? That the methods such as we and others have used in anaesthetized animals, and that which Kasé (1952, 1955) has used in conscious dogs, are perhaps to be preferred to those of Winter and Flataker (1954) is indicated by the controlled experiments of Gravenstein, Devloo, and Beecher (1954), which show that in man only relatively high doses of analgesics suppress pathological or chemically induced cough, and that moderate doses of narcotine and nalorphine are not cough suppressants.

The cough suppressant action of pholcodine has been distinguished from that of morphine-like analgesics by its relatively greater effect on "expiratory" than on "inspiratory" efforts (May and Widdicombe, 1954). The failure of nalorphine to antagonize its effects is further evidence of a different mode of action. By the same means, the action of narcotine has been distinguished from that of morphine-like compounds.

Summary

1. The antitussive actions of intravenous morphine, codeine, pethidine, methadone, piperidylamidone, thiambutene, pholcodine, and narcotine have been compared on coughs caused by electrical stimulation of the superior laryngeal nerve in anaesthetized cats. Codeine was also tested on mechanically and chemically induced coughs in anaesthetized cats, guinea-pigs, and dogs, and on a natural cough in a dog. Nalorphine was tested alone and in combination with the morphine-like drugs.

2. Methadone was the most powerful suppressant of coughs caused by the electrical stimulus in cats; its activity was slightly greater than that of piperidylamidone, about eight times that of morphine, twenty times that of pethidine, and eighty times that of codeine in cats. Pholcodine was slightly more active than codeine. Narcotine was relatively ineffective.

3. Suppression of the cough reflex by codeine and narcotine did not seem to be dependent on the type of stimulus; nor did cats, dogs, and guinea-pigs show great species variation in sensitivity to these drugs.

4. The suppressant doses of the analgesics were of the same order of magnitude as those known to cause other morphine-like effects. Relative to its antitussive action, morphine had the greatest, and pethidine the least, effect on breathing in the cat.

5. Nalorphine itself had no effect on the cough reflex, but readily abolished the suppressant action of morphine-like analgesics; similarly *N*-propylnormorphine eliminated the effect of codeine. The actions of pholcodine and narcotine were not antagonized by nalorphine.

6. The results obtained by these methods are compared with those from other experimental procedures.

7. Acute tolerance rapidly developed to the respiratory depressant action of codeine in the cat.

The authors are greatly indebted to Dr. A. C. White for valuable discussion.

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EFFECTS OF TRANSIENT CHANGES OF ACIDITY ON THE ISOLATED RAT'S UTERUS, WITH REFERENCE TO THE ASSAY OF OXYTOCIC ACTIVITY

BY

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For the biological assay of oxytocin, the *British Pharmacopoeia* (1953) recommends that the material to be tested should be washed with "one half as many ml. of a mixture of 0.25 ml. of glacial acetic acid and sufficient water to produce 100 ml. as there are Units present in the quantity of powder." and that it should be diluted so as to obtain responses similar to those from the standard preparation. These recommendations, however, are designed for estimating oxytocic activity in commercial batches of pituitary hormones and cannot be followed when estimating the oxytocic activity of extracts of pituitary glands which contain only a few milli-units of oxytocic activity.

When estimating the oxytocic activity of pituitary glands of frogs, it was found that the addition of acetic acid extracts of pituitary glands to the bath produced marked changes in the sensitivity of the uterus. The aim of the present investigation was to elucidate the causes of these changes and to examine the validity of assays when estimating similar small amounts of oxytocic activity.

METHODS

Pituitary glands of frogs were dried with acetone and each extracted with 1 ml. of a 0.25% acetic acid solution (Levinsky and Sawyer, 1953).

For the assays a strip of the horn of a rat's uterus in dioestrus was suspended in a 3 ml. bath containing a modified Locke solution (García de Jalón, Bayo Bayo, and García de Jalón, 1945) at 29.5° C. and gassed with either O_2 or a mixture of 95% O_2 and 5% CO_2 (Burn, Finney, and Goodwin, 1950). The *p*H of the modified Locke solution, measured with glass electrodes, varied between 7.4 and 7.5.

The assays were of the standard (2 + 2) dose design (*cf.* Holton, 1948); the ratio of high to low dose was the same for standard and unknown. Each dose was given once in each group of four doses and its position within the group was decided at random. Doses were added at intervals of 3 to 5 min., depending on the rate of recovery of the muscle. After the uterus had contracted, the bath was emptied and immediately refilled with the modified Locke solution. Results are given as means with their standard deviations (S.D.) and standard errors (S.E.). Significance of differences between means were determined by Students' *t* test. The fiducial limits of errors were calculated according to the *British Pharmacopoeia* (1953).

To avoid variation of the ionic composition of the bath, all dilutions were made with a 0.9% NaCl solution (van Dyke and Hastings, 1928).

Pitocin (Parke, Davis & Co.) was used as the oxytocin standard.

RESULTS

Preliminary experiments showed that, after *undiluted* doses (ranging from 0.1 to 0.2 ml.) of acetic acid extracts of posterior pituitary glands of frogs, the uterus underwent a series of spontaneous and rapid contractions. Subsequent additions of the undiluted extract resulted eventually in a complete loss of sensitivity of the preparation. This loss of sensitivity was not observed when assaying extracts which had been diluted at least 10 times with a 0.9% NaCl solution. This, however, so reduced the oxytocic activity of the extract that no quantitative estimation was possible.

To see whether both loss of sensitivity and spontaneous contractions produced by the undiluted extract were due to the extract as such or to the presence of acetic acid, two equi-active solutions of oxytocin, one with and the other without acetic acid, were compared. They were made up as follows: (*a*) 1,000 mU. of oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with a solution of 0.25 ml. glacial acetic acid in 100 ml. isotonic NaCl solution; (*b*) 1,000 mU. of oxytocin in 100 ml. of 0.9% NaCl solution. As the two preparations had the same oxytocic activity, it was expected that equal doses of either would produce contractions of comparable magnitude. It was found, however, that when the aqueous solution

was given *after* the acetic acid solution the contraction of the uterine strip was always markedly increased. In other words, the response to a constant amount of oxytocin depended upon whether it was given before or after an equal dose of acetic acid-oxytocin solution. This is illustrated in the experiment of Fig. 1, where, as the doses were given at 4 min. intervals, the sensitizing effect lasted some 30 min. After recording normal contractions of the uterus to standard doses of oxytocin, 0.1 ml. of a 0.25% acetic acid solution diluted 10 times with normal saline had no effect on the uterus (Fig. 2). It was left in contact with the tissue for periods of from 1 to 4 min., and then washed away. Subsequent standard doses of oxytocin produced contractions of the uterus significantly greater and more rapid than those recorded before the acetic acid treatment. The intensity of these changes was correlated with the duration of contact between the uterus and the acetic acid solution (Fig. 2).

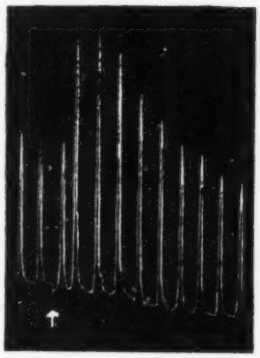

FIG. 1.—Rat uterus in 3.0 ml. bath. Effect of 0.1 ml. acetic acid-oxytocin solution (at ↑) on subsequent doses (0.1 ml. = 1.0 mU.) of standard solution of oxytocin. The acetic acid-oxytocin solution remained in the bath for 2 min. 25 sec.

To find which of the two ions, hydrogen or acetate, was responsible for the sensitization of the uterus, 0.0044 N-sodium acetate and 0.0044 N-hydrochloric acid were tested. Both solutions are equivalent to a 0.025% acetic acid solution (0.025% acetic acid solution = 0.0044 N). The addition of 0.2 ml. of the sodium acetate solution had no effect on the sensitivity of the uterus. When, however, similar doses of the 0.0044 N-HCl had been added to the bath, the uterus became more sensitive to oxytocin (Fig. 3). This showed clearly that the sensitization was the result of a change of the hydrogen ion concentration. This was confirmed by *p*H estimations: 0.1 or 0.2 ml. of either 0.025% acetic acid or 0.0044 N-HCl produced a marked drop of the *p*H to 7.2–7.1 after 2 min. exposure and subsequent removal. The difference between the falls of *p*H when the bath was gassed with O_2 or with a mixture of 95% O_2 and 5% CO_2 was not statistically significant. No significant changes in *p*H were observed when the dilution of either acid solution was increased up to 10 times or when a solution of oxytocin was given alone. Addition of 0.025 ml. 0.1 N-sodium hydroxide in a 0.9% NaCl solution, which increased the *p*H of the bath to between 8.1 and 8.3, had no significant effect on the response of the uterus to subsequent doses of oxytocin.

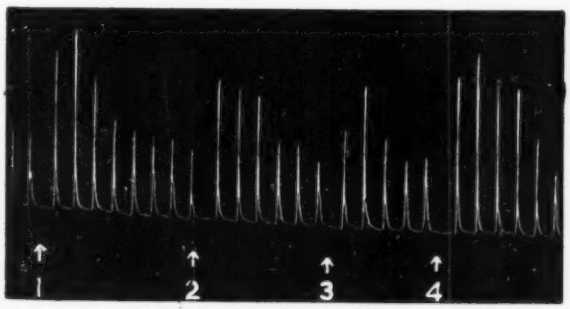

FIG. 2.—Effect of doses of 0.025% (= 0.0044 N) acetic acid on subsequent responses of rat uterus (in 3.0 ml. bath) to oxytocin. At 1, 0.2 ml. for 2 min.; at 2 and 3, 0.1 ml. for 2 min.; at 4, 0.1 ml. for 4 min. Addition of acetic acid itself had no effect on the uterus, but sensitized it to subsequent doses of oxytocin. Dose of oxytocin soln. 0.1 ml. = 1.0 mU.

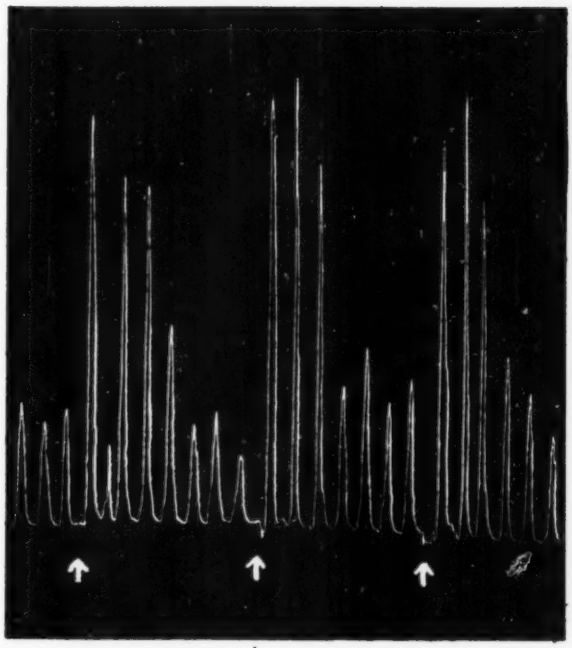

FIG. 3.—Responses of rat uterus, in 3.0 ml. bath, to 0.1 ml. (= 1.0 mU.) doses of oxytocin soln. At ↑, 0.1 ml. 0.0044 N-HCl had in itself no effect on the uterus, but sensitized it to the oxytocin.

Changes in the sensitivity of smooth muscle to drugs concomitant with changes of *p*H values have been attributed to variations in the amount of Ca ion available (Evans and Underhill, 1923; Hastings, Murray, and Sendroy, 1927; Kerridge and Winton, 1929). Raising the normal $CaCl_2$ concentration of the bath fluid from 0.06 g./l. to 0.09 and 0.12 g./l.

produced, as a rule, *one* marked spontaneous contraction of the uterus, confirming van Dyke and Hastings' (1928) findings on the guinea-pig uterus. However, as soon as the extra Ca had been washed away, there was no increased sensitivity to oxytocin.

The modified Locke solution, buffered with sodium bicarbonate, was replaced in 6 experiments by a modified Ringer-Evans solution containing a phosphate buffer. Its composition was: NaCl 9.00 g.; KCl 0.42 g.; $CaCl_2$ 0.06 g.; Na_2HPO_4 0.6 g.; 1M-H_3PO_4 0.2 to 0.6 ml.; glucose 0.5 g., made up to 1,000 ml. with distilled water. The presence of a phosphate buffer decreased the sensitivity of the uterus to oxytocin. Addition of 0.2 ml. of 0.25% acetic acid, diluted tenfold with a 0.9% NaCl solution, produced only a transient increase in sensitivity to oxytocin; the response to a second or third dose of oxytocin was always similar to that before the acetic acid solution. Estimations of *p*H values showed that with this Ringer solution the acetic acid produced falls which were not significant; they did not exceed 0.1.

In view of these changes in sensitivity of the uterine preparation, the validity of assays of acid extracts that for practical reasons could not be greatly diluted was examined. Estimations of both "known" and "unknown" solutions of the same concentration of oxytocin were attempted first. The "unknown" solution was divided into halves; one without acetic acid, identical with the known solution, was called "saline unknown"; it contained 750 or 1,000 mU. oxytocin/100 ml. of a 0.9% NaCl solution. The composition of the other half was 750 or 1,000 mU. oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with 0.25% acetic acid solution. This was the "acetic unknown." This concentration of acetic acid is 20 times greater than that recommended by the *British Pharmacopoeia* but 2.5 times less than that used by Levinsky and Sawyer (1953).

TABLE I

(2+2) DOSE ASSAYS OF KNOWN SOLUTIONS OF OXYTOCIN

Description of Unknown Solutions	No. of Assays	Mean Amount of Oxytocic Activity (mU.)	S.E. ±	S.D. ±
1,000 mU. oxytocin in 100 ml. 0·9% NaCl (1) 1·0 ml. = 10·0 mU.	10	9·9	0·17	0·53
1,000 mU. oxytocin in 90 ml. 0·9% NaCl + 10 ml. of 0·25% acetic acid (2) 1·0 ml. = 10·0 mU.	7	8·6	0·78	2·06
750 mU. oxytocin in 100 ml. 0·9% NaCl (3) 1·0 ml. = 7·5 mU.	10	7·4	0·26	1·67
750 mU. oxytocin in 90 ml. 0·9% NaCl + 10 ml. of 0·25% acetic acid (4) 1·0 ml. = 7·5 mU.	9	6·0	0·55	0·94

All standard solutions were 1·0 ml. = 10·0 mU. oxytocin.
For (1) and (2), $t = 2{\cdot}017$, $P < 0{\cdot}1 > 0{\cdot}05$ ($n = 15$).
For (3) and (4), $t = 2{\cdot}298$, $P < 0{\cdot}05$ ($n = 17$).

In each assay two doses of "unknown" were compared with two doses of "known"; each estimation consisted of 4 groups of 4 randomized injections. The results of 36 such assays are given in Table I. The two "saline unknowns," containing 7.5 and 10 mU./ml. respectively, assayed at 7.4 ± 0.26 and 9.9 ± 0.7 mU./ml. But the two "acetic unknowns" of identical potency assayed at 6.0 ± 0.55 and 8.6 ± 0.78 respectively. At each concentration there was a significant difference ($P = 0.05$) between the mean estimated potency of "saline unknowns" and "acetic unknowns."

It is not always possible for practical reasons to have the adequate quantity of material necessary for a (2+2) dose assay. In those circumstances, it might be tempting to compare the effect of one dose of "acetic extract" with two different doses of standard in a (2+1) dose assay. It was, however, impossible to estimate the potency of an "acetic unknown" by "matching" its effect with that of two equal doses of the standard, as the reaction of the uterus to the dose of standard given after the "unknown" was significantly greater than that observed for the same dose of standard given before the "unknown." To avoid this difficulty an "acetic unknown" containing 7.5 mU. oxytocin/ml. (750 mU. oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with 0.25% of acetic acid) was compared with two different doses of standard, 1.0 and 0.5 mU. oxytocin. The "acetic unknown" was bracketed by the two different standards. Theoretically, the reaction of the uterus to 0.1 ml. of the "acetic unknown" should have been equal to the mean of the effects of the two doses of the standard. The results were, however, quite unpredictable. Nine out of eighteen estimations had to be rejected, since the "unknown" failed to fall between the two doses of standard. The range of the remaining 9 estimations, calculated according to Gaddum (1954), varied from 5.3 to 9.1 with a mean of 5.5 ± 0.83 mU. oxytocin/ml. This showed conclusively that no reliable information about the amount of oxytocin activity of a preparation in which the acetic acid was not adequately diluted could be obtained from assays in which the "unknown" was tested at one dose level only.

Discussion

Evans and Underhill (1923) have shown that small amounts of acid enhanced the reactions of the guinea-pig's uterus to posterior pituitary extracts, and this was confirmed on the rat. On the other hand, according to Hemingway (1926), raising the *p*H values of the bath from 7.4 to 8.5 increased the sensitivity of the cat's uterus both to histamine and to posterior pituitary lobe extract. Similar changes in *p*H, however, had no effect on the sensitivity of the rat's uterus.

At *p*H values of 7.0 to 8.0 the buffer capacity of sodium bicarbonate of the modified Locke solution is very poor, since in *in vitro* experiments no regulation of the carbon dioxide levels can obtain (Ling and Smith, 1955). Hence, it was not surprising to find that the addition to the bath of small amounts of a 0.025% acetic acid solution produced significant changes in *p*H. These changes significantly enhanced the response of the rat's uterus to oxytocin; and though the increase of the hydrogen ion concentration was transient, its effect on the uterus lasted up to 30 min. These increased responses did not occur, however, when the acetic acid had been adequately diluted, for example, 100 times.

These findings had a definite bearing on the accuracy of assays of solutions of oxytocin of known concentration made up with 0.25% acetic acid solution tenfold diluted, to imitate a tissue extract of low oxytocic activity. In 16 such assays (Table I), comprising 4 groups of each of 2 unknown and 2 standard, the mean amount of oxytocic activity was found to be about 14% lower than that of a similar solution without acetic acid. From this it would be logical to assume that a transient increase in acidity of the bath may affect the accuracy of assays of posterior pituitary gland extracts, which, on account of their initial low oxytocic activity, cannot be greatly diluted. According to the *British Pharmacopoeia* (1953) the dry powder obtained after extraction of the posterior pituitary gland with acetone should be washed with one half as many ml. of a mixture of 0.25% acetic acid solution as there are *Units* present in the quantity of powder. This can be easily done when dealing with pituitary glands of adult mammals, such as the dog (Dicker and Tyler, 1953a), but not with individual glands of amphibians, young mammals or foetuses, as these contain only a few milli-units of oxytocic activity. The following amounts of 0.25% acetic acid solution have been used; amphibians, 1.0 ml./gland (Heller, 1941; Levinsky and Sawyer, 1953); newborn rats, 0.3 ml./gland (Heller, 1947); newborn infants, 0.5 ml./mg. of dry gland tissue (Heller and Zaimis, 1949); puppies, kittens, newborn rats, newborn guinea-pigs and human foetuses, 1.0 ml./gland (Dicker and Tyler, 1953a and b). When related to their oxytocic activity these amounts were far in excess of those used for adult mammals (Dicker and Tyler, 1953a), or recommended by the *British Pharmacopoeia* (1953). It is clear from the present investigations that unless diluted between 100 to 200 times, the high hydrogen ion concentration of such extracts will increase the normal reactions of the rat's uterus to oxytocin and hence impair the reliability of the assays. Adequate dilutions would, however, be impracticable, as their oxytocic activity would be too small to be estimated. Heller (1947), Heller and Zaimis (1949) and Dicker and Tyler (1953a and b) used extracts which were diluted between 3 and 5 times only, whereas Levinsky and Sawyer (1953) tested undiluted preparations. To overcome this difficulty, it might be advisable to pool as many glands as possible. It would need the pooling of 10 to 15 glands of frogs, puppies, or kittens to have enough oxytocic activity in the extract to allow it to be diluted accurately, i.e., to have enough material to test at an appropriate hydrogen concentration. This, however, would be practically impossible with neurohypophyses of embryos and especially of human foetuses (Dicker and Tyler, 1953b).

Summary

1. Estimations of the oxytocic activity of frogs' neurohypophyses were attempted on an isolated rat's uterus preparation.

2. Addition of dilute acetic acid extracts sensitized the preparation to subsequent doses of aqueous extracts.

3. The sensitization, which lasted some 30 min., was not due to the tissue extract, but was the result of a transient increase of the hydrogen ion concentration.

4. The validity of assays of acetic acid extracts of individual neurohypophyses, which on account of the paucity of their oxytocic activity could not be diluted adequately, is discussed and criticized.

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SIDE EFFECTS OF CHLORPROMAZINE HYDROCHLORIDE

BY

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Chlorpromazine hydrochloride (3-chloro-10(3′-dimethylamino-*n*-propyl) phenothiazine hydrochloride) is related to promethazine hydrochloride and to various compounds used in the treatment of Parkinson's disease. It has been claimed to have an analgesic action (Sigwald and Bouttier, 1953 ; Howell, Harth, and Dietrich, 1954), and was shown to prevent vomiting caused by apomorphine (Isaacs and MacArthur, 1955), and in other ways (Moyer, Kent, Knight, Morris, Dizon, Rogers, and Spurr, 1954). Promethazine has a hyoscine-like action (Bain, 1949 ; Glaser, 1953 ; Whittow, 1954), and since this probably plays a part in the treatment of Parkinson's disease and of vomiting, it was of interest to find out whether chlorpromazine exerted a hyoscine-like action on healthy people.

Previous investigators concerned with side effects of drugs have shown that habituation may take place to the taking of tablets and the filling-in of questionnaires (Glaser, 1953 ; Glaser and Whittow, 1953, 1954), and it seemed probable that this habituation had its origin in the cerebral cortex (Glaser and Whittow, 1953). Since chlorpromazine exerts a depressant action on the highest centres of the brain (Sigwald and Bouttier, 1953), it seemed possible that it would inhibit habituation, and the present experiments offered an opportunity to find out whether this was so. (Habituation is taken to mean a gradual diminution of the response to a repeated or continued stimulus, taking place in periods of time of the order of days ; Glaser, 1955.)

METHODS

The experiments were conducted according to a procedure previously described (Glaser, 1953, 1955) in which all the substances to be tested, as well as an inert substance, are given as indistinguishable tablets at the same time of the day at intervals of 7 days and in a random sequence to all subjects, so that each subject receives every substance and each substance is given to a similar number of subjects each time. The tablets are issued according to a code, and the experimenters do not have the key to this code until the results have been worked out. The subjects of the experiment do not know what substances are to be tested, nor do they know whether the same substance or different substances are given each time. The results are recorded on carefully designed questionnaires about 1½ hr. after the substances have been taken. The validity of this procedure was confirmed in experiments designed to estimate its errors (Glaser, 1954 ; Glaser and Whittow, 1954).

Two experiments were conducted on two different groups of subjects. In one experiment 50 students were given indistinguishable sugar-coated tablets, containing either 25 mg. chlorpromazine hydrochloride or lactose, so that half the subjects took the drug first and half the subjects took the lactose first. In the other experiment 80 students were given 50 mg. chlorpromazine hydrochloride, 25 mg. chlorpromazine hydrochloride, 0.75 mg. hyoscine hydrobromide or a lactose dummy in sugar-coated tablets, as described above, so that each kind of tablet was preceded by every other kind of tablet in an equal number of tests. There was no obvious self-selection among the subjects, since the number of students who were able to volunteer for the experiment but did not do so was less than 5%. The questionnaires were similar to those previously described (Glaser, 1953 ; Glaser and Whittow, 1954), but the subjects were also asked to record their pulse rates and, in the experiment on 50 subjects, to record their own mouth temperatures with numbered thermometers, which they placed in their mouths simultaneously for 3 min. The thermometers were then collected and the readings which had been recorded were checked.

Associations between the incidence of various symptoms were calculated by 2 × 2 tables using the χ^2 method and applying Yates's correction for continuity, while the significance of differences between pulse rates was assessed by means of Student's t test.

RESULTS

In the experiment in which 25 mg. chlorpromazine hydrochloride was compared with a dummy tablet the only significant difference was that chlorpromazine was considered unpleasant by 11 subjects and the dummy by 3 ($\chi^2=4.070$, $P<.05$). The mean pulse rate was 85 after taking chlorpromazine and 81 after taking the dummy ($.05>P>.02$), but the mouth temperatures were the same.

* Medical Research Council Scientific Staff.

The results of the experiment in which 80 subjects were given in turn a dummy substance, 25 mg. chlorpromazine, 50 mg. chlorpromazine, and 0.75 mg. hyoscine are shown in Table I. The statistical significance of the results is shown in Table II. The number of subjects who took each drug varied, because some students missed certain classes and therefore missed taking certain drugs, but the proportion of absentees was never more than 5%,

TABLE I

EFFECTS OF VARYING DOSES OF CHLORPROMAZINE HYDROCHLORIDE, COMPARED WITH THE EFFECTS OF HYOSCINE HYDROBROMIDE AND A DUMMY TABLET

Symptom	Dummy	Chlorpromazine		Hyoscine 0·75 mg.
		25 mg.	50 mg.	
Headache	18	15	19	28
Sleepiness	23	31	50	54
Tiredness	20	29	44	43
Flushing of face	12	11	13	19
Nausea	12	12	27	36
Inability to think clearly	9	12	20	34
Dryness of mouth	30	47	51	62
Backache	10	10	18	14
Giddiness	15	10	27	47
Faintness	5	4	15	29
Feeling unwell	5	9	23	28
Hunger	19	24	21	22
No. of symptoms recorded by all subjects	178	214	328	416
No. of subjects showing any effect	62	58	70	71
Substance considered pleasant	7	3	6	4
Substance considered unpleasant	12	22	32	45
Mean pulse rate	81	81	90	76
No. of subjects taking tablet	78	76	76	77

and it could not have invalidated the results. Chlorpromazine (25 mg.) again produced results which did not differ much from those obtained with the dummy, except that in this group of subjects dryness of the mouth was significantly more frequent after the chlorpromazine than after the dummy, and the number of subjects who considered 25 mg. chlorpromazine unpleasant was not significantly greater than the number who considered the dummy unpleasant ($\chi^2=3.365$, $.10>P>.05$). Both 50 mg. chlorpromazine and 0.75 mg. hyoscine produced significantly more symptoms than did either 25 mg. chlorpromazine or the dummy. The incidence of symptoms was somewhat higher after hyoscine than after 50 mg. chlorpromazine, but only inability to think clearly, faintness, and giddiness were significantly more frequent after hyoscine than after 50 mg. chlorpromazine. The total number of symptoms recorded by all subjects, however, was also significantly higher after hyoscine than after chlorpromazine ($\chi^2=14.723$; $P<.01$). The mean pulse rate in this group of subjects was the same after 25 mg. chlorpromazine and after the dummy, but it was significantly higher after 50 mg. chlorpromazine than after the dummy ($t=3.6443$; $P<.01$), and it was significantly lower after 0.75 mg. hyoscine hydrobromide than after the dummy ($t=3.2036$; $P<.01$).

TABLE II

STATISTICAL SIGNIFICANCE OF DIFFERENCES GIVEN IN TABLE I

Only significant differences are shown ($P<.05$). Highly significant differences ($P<.01$) are marked *

Substances Compared	Symptom	χ^2
Chlorpromazine 25 mg. —lactose dummy	Dryness of mouth	7·508*
Chlorpromazine 50 mg. —lactose dummy	Sleepiness	18·916*
	Tiredness	15·187*
	Nausea	7·227*
	Inability to think clearly	4·575
	Dryness of mouth	11·544*
	Giddiness	4·833
	Faintness	4·928
	Feeling unwell	13·163*
	Substance considered unpleasant	12·190*
Hyoscine—lactose dummy	Sleepiness	24·003*
	Tiredness	13·427*
	Nausea	16·398*
	Inability to think clearly	18·970*
	Dryness of mouth	26·694*
	Giddiness	26·505*
	Faintness	20·314*
	Feeling unwell	18·997*
	Substance considered unpleasant	29·072*
	Number of subjects showing effect	4·156
Chlorpromazine 50 mg.	Sleepiness	8·563*
,, 25 mg.	Tiredness	5·166
	Nausea	6·760
	Giddiness	9·145*
	Faintness	6·015
	Feeling unwell	6·690*
	Number of subjects showing effect	5·986
Hyoscine—chlorpromazine 25 mg.	Headache	4·443
	Sleepiness	12·173*
	Tiredness	4·119
	Nausea	15·624*
	Inability to think clearly	13·319*
	Dryness of mouth	5·633
	Giddiness	35·492*
	Faintness	21·857*
	Feeling unwell	11·242*
	Substance considered unpleasant	12·346*
	Number of subjects showing effect	6·152
Hyoscine—chlorpromazine 50 mg.	Inability to think clearly	4·578
	Giddiness	8·973*
	Faintness	5·156

Fig. 1 shows the frequency distribution of symptoms after each of the four tablets given to 80 subjects. Although the distribution was not binomial, it is evident that the response to 25 mg. chlorpromazine closely resembled the result obtained with the dummy, while the response to 50 mg. chlorpromazine closely resembled that

with hyoscine. This further confirms the results given above.

Further analysis of the results showed that certain subjects who had reported symptoms of one kind or another had answered the question whether they felt unwell in the negative. Fig. 2 compares the number of symptoms recorded by these subjects with those recorded by subjects who considered themselves to be unwell (irrespective of substances taken), and it shows that those who thought that they were unwell recorded more symptoms (mean 8.71) than those who thought that they were well (mean 2.73). Since the relative incidence of symptoms after taking each substance was the same, whether the subjects considered themselves well or unwell, this does not affect the above conclusions, but it suggests that the subjects fell into two distinct groups with regard to the number of symptoms they reported, and that those who tended to report more symptoms could be picked out by a tendency to consider themselves unwell. This opens up possibilities for research on individual susceptibilities.

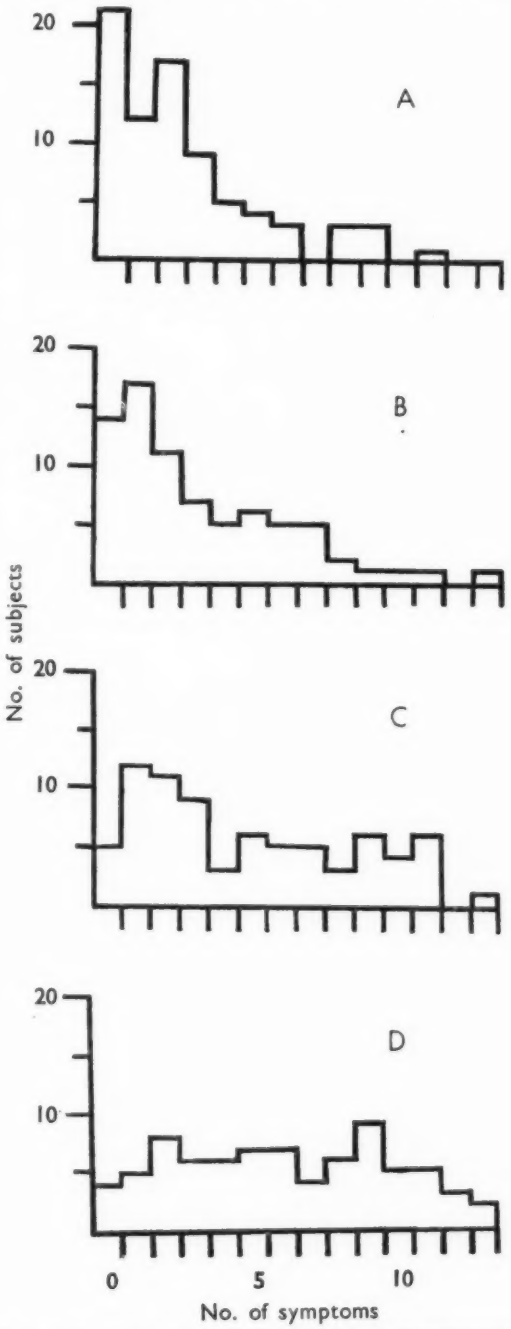

FIG. 1.—Frequency distributions of the number of symptoms reported after taking a dummy tablet (A), chlorpromazine hydrochloride (B, 25 mg., and C, 50 mg.) and hyoscine hydrobromide (D, 0·75 mg.). Number of subjects, 80.

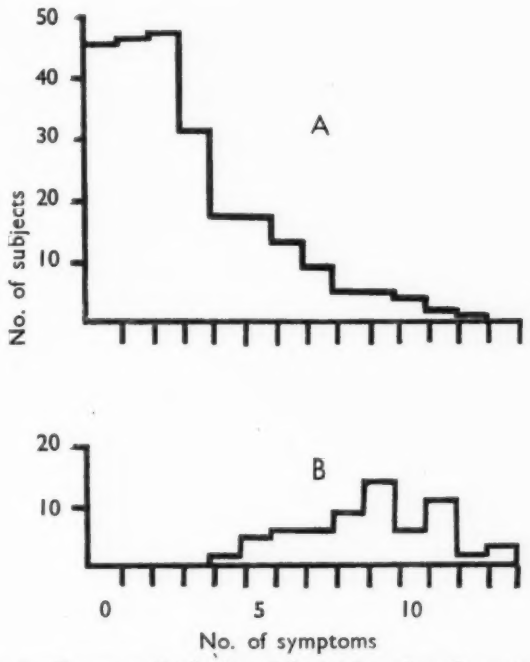

FIG. 2.—Frequency distribution of the total number of symptoms recorded by subjects who considered themselves to be well and subjects who considered themselves to be unwell. A, well; B, unwell. Number of subjects, 80.

Comparison of the results obtained with 0.75 mg. hyoscine hydrobromide in the present experiment (Table I) and with 1 mg. of the same drug in a similar experiment (Glaser, 1953, Table I) showed that the differences between the effects of both doses were compatible with random differences between two identical samples. This conforms with previous findings at sea in which no significant difference was found between the effects of 0.6 and 1.2 mg. hyoscine hydrobromide (Holling,

McArdle, and Trotter, 1944), and it suggests that the optimal dose of this drug is about 0.6–0.75 mg.

Habituation.—Seven days after the cross-over experiment with 4 tablets, each subject was given the same type of tablet as he had received on the first day of the experiment, while all other procedures remained unchanged. Only 62 of the subjects were available to repeat the first test and their responses at the beginning and end of the experiment are compared in Tables III and IV.

TABLE III
SYMPTOMS RECORDED AFTER GIVING THE SAME SUBSTANCES TO THE SAME SUBJECTS IN DIFFERENT TESTS (62 subjects)
Where the difference is significant, the numbers are underlined

Symptom	No. of Subjects Showing Symptom	
	1st Test	5th Test
Headache	25	15
Sleepiness	38	34
Tiredness	36	31
Flushing of face	15	8
Nausea	23	18
Inability to think clearly	18	16
Dryness of mouth	41	34
Backache	10	8
Giddiness	35	18
Faintness	15	12
Hunger	15	12
No. of symptoms	271	206
Substance considered unpleasant	31	26

TABLE IV
EFFECTS OF GIVING THE SAME SUBSTANCE TO THE SAME SUBJECTS IN DIFFERENT TESTS

Substance	No. taking Substance	Total No. of Symptoms		χ^2	P
		1st Test	5th Test		
Lactose dummy	15	49	28	6·775	0·01
Chlorpromazine, 25 mg.	14	49	31	4·820	0·02–0·05
Hyoscine	15	96	77	3·936	0·05
Chlorpromazine, 50 mg.	18	77	70	0·389	0·50–0·70

All symptoms were less frequent when substances were given for the second time than when they were given for the first time, but giddiness was the only symptom which decreased significantly ($\chi^2=8.443$; $P<.01$), while the decrease in the incidence of headaches was approaching statistical significance ($\chi^2=3.642$; $P<.05$). The total number of symptoms recorded by all subjects, however, decreased by a highly significant amount ($\chi^2=13.204$; $P<.01$). Table IV shows that this decreased incidence of symptoms was unevenly distributed among the various substances. The decrease in the total number of symptoms recorded after the dummy for the second time amounted to 42%, and it was highly significant, whereas the decrease in the number of symptoms after 25 mg. chlorpromazine was only slightly smaller. The decrease after hyoscine hydrobromide was only just significant. After 50 mg. chlorpromazine, however, the decrease of symptoms between the first and the fifth day of the experiment was insignificant. This suggests that habituation to the experiment had taken place, but that this habituation was inhibited by 50 mg. chlorpromazine.

DISCUSSION

It seems evident from the above results that 50 mg. chlorpromazine has an action which resembles that of 0.75 mg. hyoscine hydrobromide, but chlorpromazine accelerates the heart rate, whereas hyoscine slows it. Chlorpromazine in doses of 50 mg. must also have some action on the cerebral cortex, since it clearly inhibited habituation to the experiment, and this conforms with reports of its value in the treatment of psychiatric conditions and in anaesthesia. Twenty-five mg. of chlorpromazine has very little effect on healthy people, but it is not entirely inert when compared with lactose.

Previous experiments have shown that drugs which have a hyoscine-like central action on dry land are effective in the prevention and treatment of motion sickness (Glaser, 1953, 1955), and it has been suggested that side effects of drugs may give some indication of their effectiveness against motion sickness (Whittow, 1954). The present results suggest, therefore, that 50 mg. chlorpromazine might be a useful preventive for motion sickness. The findings of Isaacs and MacArthur (1955) that such a dose of chlorpromazine was effective against vomiting induced by emetine are not conclusive, owing to the possibility that the central nervous mechanism of vomiting caused by emetine differs somewhat from that of vomiting in motion sickness (Borison and Wang, 1953). Handford, Cone, Chinn, and Smith (1954) have, in fact, found 50 mg. of chlorpromazine ineffective during prolonged exposures to sea-sickness. The effectiveness of remedies for motion sickness depends, however, to some extent on the kind of motion and on the state of adaptation of the patients (Glaser, 1955), so that it is possible that chlorpromazine might be effective against motion sickness in some circumstances.

The inhibition of habituation by chlorpromazine requires confirmation, but it offers some hope that chlorpromazine may provide a tool for the study of the physiological basis of this phenomenon.

SUMMARY

1. Twenty-five mg. chlorpromazine had little effect on healthy people.

2. Fifty mg. chlorpromazine produced effects similar to those of hyoscine, including sleepiness, tiredness, and dryness of the mouth, but it increased the pulse rate.

3. There was some evidence that 50 mg. chlorpromazine inhibited habituation to experimental procedures, and this indicates a depressant action on the cerebral cortex.

We are indebted to Messrs. May & Baker, Ltd., of Dagenham, Essex, for providing us with all the indistinguishable tablets used in these experiments.

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Errata

Glaser, E. M., and Newling, P. S. B., vol. 10, page 432, second para. of discussion:
Line 11, *for* (1955) *read* (1954).
Line 12, *for* emetine *read* apomorphine.

Brit. J. Pharmacol. (1955), **10**, 434.

THE AMOEBICIDAL ACTIVITY OF 2-DIETHANOLAMINO-5-NITROPYRIDINE

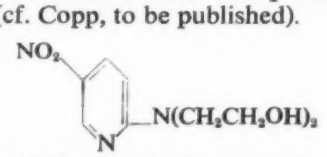
BY

R. A. NEAL AND PATRICIA VINCENT

From the Wellcome Laboratories of Tropical Medicine, London

(RECEIVED JUNE 22, 1955)

During the examination of a miscellaneous collection of chemicals for amoebicidal activity, 2-diethanolamino-5-nitropyridine (263C49) showed marked activity associated with low toxicity. Activity against *Entamoeba histolytica* by this type of chemical structure has not previously been described (cf. Copp, to be published).



2-diethanolamino-5-nitropyridine

MATERIAL AND METHODS

Caecal Infection in the Rat.—Young rats weighing less than 35 g. were infected by injecting cultured amoebae directly into the caecum, exposed at laparotomy. The rats were then given six daily doses of drug by mouth, the first being administered on the day following inoculation, and were finally killed and examined on the seventh day. Drugs were given in solution or suspended with compound powder of tragacanth in distilled water. The extent of caecal ulceration was given a score, the condition of the wall (normal 0, extensive ulceration 4) being scored separately from the contents (normal 0, mucus only 4), thus making a maximum score of 8 for each animal (cf. Neal, 1951). The caecal contents were examined microscopically for *E. histolytica*; if none was seen the contents were cultured in a medium consisting of an inspissated horse-serum slope covered by egg-white diluted with Ringer solution plus rice starch. The cultures were examined after 24, 48, and sometimes 72 hours' incubation at 37° C. Three strains of *E. histolytica* were used, one invasive (*M*) and two (*STC* and *EA*) which rarely invaded the caecal wall.

Liver Abscess in the Hamster.—The technique for determining the effect of the drug on liver abscesses was similar to that described by Thompson and Reinertson (1951). About 0.1 to 0.15 ml. of a suspension of amoebae from culture containing at least 20,000 amoebae was injected directly into the livers of hamsters exposed under hexobarbitone anaesthesia. The hamsters were given an oral dose of drug on each of the following 4 days and killed 7 days after inoculation. At autopsy the lesion was removed and weighed, the presence of amoebae being determined by microscopical and cultural examination. The strain used for producing liver abscess was a derivative of *M*, which had been serially passaged in the livers of hamsters on five occasions.

In vitro *Experiments.*—Investigation of the activity *in vitro* was made with two pure strains of *E. histolytica*, $P + A^1$ and $D + R^4$ (amoebae plus *Escherichia coli*). The drug and bacteria were added to the medium and incubated overnight; the amoebae were added the following day (Dobell, 1947). The tubes were examined microscopically for the presence of amoebae after 3 days' incubation at 37° C. The media used for the *in vitro* tests were dilute horse serum with 10% liver extract for $P + A^1$, and dilute horse serum with 0.1% dextrose for $D + R^4$. Rice starch was added to each medium.

RESULTS AND DISCUSSION

A comparison of the amoebicidal activity of 263C49 and chiniofon upon the caecal infection of *Entamoeba histolytica* in rats is shown in Table I.

TABLE I

EFFECT OF 263C49 AND CHINIOFON ON *E. HISTOLYTICA* IN RATS

Strain	Drug	Dose (mg./kg. ×6)	Proportion of Rats Cleared of Amoebae	Mean Caecal Score
STC (non-invasive)	263C49	250	4/11	0
		125	11/23	0·1
		62·5	1/11	0·3
	Controls	—	3/21	1·0
EA (non-invasive)	263C49	500	5/7	0·3
		250	7/17	0
		125	3/10	0·6
	Chiniofon	250	7/7	0
		125	6/7	0
		62·5	6/10	0·4
		31·25	4/10	0
	Controls	—	10/36	0·6
M (invasive)	263C49	250	5/6	0·3
		125	10/15	0·3
		62·5	1/7	2·4
	Chiniofon	250	5/6	0·3
		125	9/16	1·3
		62·5	0/7	5·6
	Controls	—	2/13	5·4

Against the invasive strain *M*, 263C49 has the same activity as chiniofon. The caecal scores show that the lowest dose of 263C49 (62.5 mg./kg.) decreased the degree of ulceration, although it did not eliminate the amoebae from the lumen. Chiniofon at this dose level had no effect on the infection rate or the extent of ulceration.

Against the non-invasive strains, 263C49 proved less effective than against the invasive strains. Chiniofon, on the other hand, was more effective on non-invasive strains than invasive strains. Esters of 263C49, which had low solubility in water, were just as ineffective upon the non-invasive strains as the parent compound. The simultaneous administration of 10,000 units per day of polymyxin B sulphate did not enhance the amoebicidal activity of 263C49.

Table II shows the effect on hepatic lesions in hamsters. Chloroquine was effective at 200 mg./kg., but 263C49 had no effect at 250 mg./kg.

TABLE II

EFFECT OF 263C49 ON AMOEBIC LIVER ABSCESS IN HAMSTERS

Expt.	Drug	Dose (mg./kg. ×4)	Proportion with Abscess	Average Lesion Wt. (g.)
1	Chloroquine	200	1/7	0·33
	263C49	250	5/8	2·36
	Controls	—	4/8	1·22
2	Chloroquine	150	5/6	1·61
		75	2/3	1·61
	263C49	150	5/6	2·51
		75	6/6	2·49
	Controls	—	3/4	1·56

In vitro, the minimum amoebicidal concentration of 263C49 was 10^{-5}, while chiniofon was effective at a concentration of 10^{-5} (strain $P+A^1$) and 10^{-4} (strain $D+R^4$). As the nitropyridine derivative showed no inhibition of the growth of *Escherichia coli* at a concentration of 10^{-4}, the drug action is probably directly upon the protozoa.

Toxicity experiments did not reveal any adverse effects on the haemoglobin, red cell, or white cell counts of rabbits. Microscopical examination of liver biopsies from these animals showed no abnormality. A group of mice received 53 daily doses of 250 mg./kg. without showing any inhibition of growth. The drug was rapidly excreted in the urine.

A small clinical trial was carried out by Dr. R. Elsdon-Dew at Durban, South Africa. He gave a very high dose, 51 g. orally over ten days, to a series of fourteen cases of amoebic dysentery and found that, though all cases were rapidly cleared of their symptoms, a proportion of them showed parasitological relapse after treatment.

The nitropyridine was tested for therapeutic effects upon laboratory infections of the following protozoa, but no activity was revealed—*Trypanosoma rhodesiense, T. congolense, T. cruzi, Leishmania donovani, Plasmodium gallinaceum,* and *Histomonas meleagridis.*

SUMMARY

1. The amoebicidal activity of 2-diethanolamino-5-nitropyridine (263C49) was equal to that of chiniofon on rats experimentally infected with an invasive strain of *E. histolytica.* 263C49 was less effective on non-invasive strains of *E. histolytica.* It had no activity on experimentally induced liver abscesses in hamsters.

2. *In vitro,* 263C49 was found to have a direct action on the amoebae at the same minimum concentration as chiniofon. On one strain, the new drug was ten times more active than chiniofon.

3. A small clinical trial showed that a very high dose cleared the clinical symptoms, but parasitological relapse occurred later.

We should like to thank Miss T. Tilley and Mr. W. H. G. Richards for technical assistance and our colleagues at the Wellcome Laboratories for testing this drug on other protozoa.

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Brit. J. Pharmacol. (1955), **10**, 436.

ALPHA-COCAINE

BY

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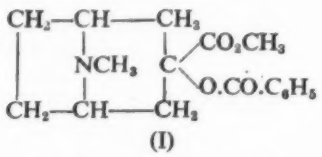
(RECEIVED JUNE 30, 1955)

Ever since Willstätter synthesized α-cocaine (I) in 1896, his statement that it did not possess local anaesthetic properties has been accepted, although his only evidence was that it did not have the numbing effect on the tongue characteristic of cocaine. α-Cocaine is a structural isomer of cocaine (II) and is closely related in structure to α-eucaine (III), one of the earliest synthetic local anaesthetics; consequently it is difficult to understand why Willstätter's statement was accepted



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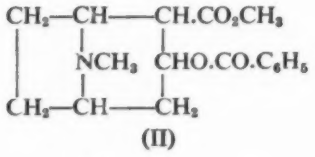

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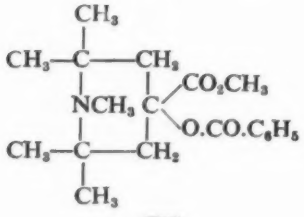

(III)

uncritically for so many years. Since tropinone, from which Willstätter synthesized α-cocaine, is now available commercially, we have repeated his synthesis and tested α-cocaine for local anaesthetic activity by other methods. α-Cocaine does, in fact, possess local anaesthetic properties, though inferior to those of cocaine. We have also investigated its ability to potentiate some of the pharmacological effects of adrenaline, as cocaine is well known to do; it was also compared with cocaine as an inhibitor of amine oxidase.

METHODS

Local anaesthetic activity was tested on the cornea of the rabbit by the method of Chance and Lobstein (1944) and also by the guinea-pig weal method and frog plexus anaesthesia method described by Bülbring and Wajda (1945). The effects of α-cocaine upon adrenaline responses were tested on the isolated uterus, the isolated auricles, and the isolated duodenum of the rabbit, on the perfused vessels of the rabbit ear, and on the blood pressure of the spinal cat. The effects of α-cocaine upon noradrenaline responses were tested on all but the first two of these preparations. α-Cocaine and cocaine were also compared as inhibitors of amine oxidase activity. This was measured by the manometric technique with the following solutions: 0.067M-phosphate buffer, *p*H 7.4; 0.01M-KCN; KOH/KCN (1 ml. N-KOH + 10 ml. N-KCN) (centre well).

The following preparations were made: cat liver was homogenized in 0.067M-phosphate buffer, *p*H 7.4, in a glass homogenizer and dialysed for 4 hours against running tap water. After dialysis one-fifth of its volume of 0.2M-phosphate buffer, *p*H 7.4, was added and the preparation stored frozen. Rabbit liver was prepared in the same way.

Rabbit uterus was finely chopped on a McIlwain chopper and suspended in 0.067M-phosphate buffer, *p*H 7.4.

Cat nictitating membrane was freed from fat, and the cartilage and Harderian gland were removed. The membrane was finely chopped and the whole mince was transferred to a manometer vessel. The oxygen uptake of R and L nictitating membranes of equal weight after dissection were compared in the presence of cocaine and α-cocaine respectively. No control observation without inhibitor was made owing to the difficulty of comparing nictitating membranes from different cats and to the difficulty of sampling pooled material.

RESULTS

Local Anaesthetic Activity. (*a*) *Rabbit Cornea.*—A comparison between the action of α-cocaine and that of cocaine was made by instilling solutions into the conjunctival sac of 13 rabbits. Cocaine was tested on one eye and α-cocaine was tested on the opposite eye. Cocaine hydrochloride was

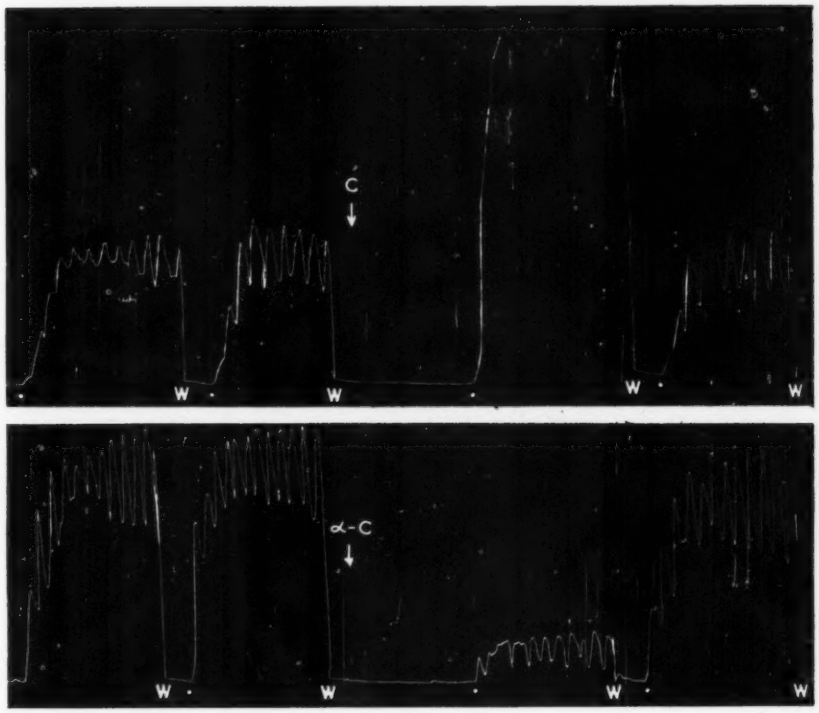

FIG. 1.—Contractions of isolated rabbit uterus in 10 ml. bath. Each white dot represents addition of 0·2 μg. adrenaline. W represents wash-out of bath. Arrow in upper tracing indicates addition of 0·2 mg. cocaine and that in the lower tracing addition of 0·2 mg. α-cocaine. Note that cocaine potentiated the effect of adrenaline, whereas the same dose of α-cocaine reduced the effect of adrenaline but did not abolish it entirely.

normally active in all the rabbits in solutions of strength 0.25–1.0% (w/v). In 3 rabbits α-cocaine was active in 1.0% solution, and in a fourth in 5% solution, but in 9 rabbits it was completely inactive, even in 5% solution.

(*b*) *Intradermal Injections in Guinea-pigs.*—In this method the injected area is tested at 5 min. intervals for 30 min., six pricks being made in each of the six tests. If there is no response to any prick the maximum score, representing full anaesthesia through the period, is 36. The results of the comparison between α-cocaine and cocaine are expressed in this way in Table I.

When the results of the two experiments were expressed graphically, it was found that 1 mg. α-cocaine was equivalent to 0.12 mg. cocaine in Expt. 1 and to 0.31 mg. cocaine in Expt. 2. The mean figure indicates that α-cocaine was approximately one-fifth as active as cocaine.

(*c*) *Plexus Anaesthesia in Frogs.*—In this method the response of the decerebrate frog to the application of dilute HCl to the skin of the hindlegs is determined and the time from the introduction of the anaesthetic solution into the abdominal cavity until the response fails is measured. The results are given in Table II, each figure for the time to failure of response being determined in 4 frogs.

TABLE I

COMPARISON OF α-COCAINE WITH COCAINE BY GUINEA-PIG WEAL TEST

Expt.	Substance	Concentration g./100 ml.	No. of Weals Tested	Degree of Anaesthesia Max. = 36
1	Cocaine	0·05	7	17
		0·1	8	24
		0·2	6	32
	α-Cocaine	0·1	2	3
		0·2	3	10
		0·35	8	13
		1·0	4	25
2	Cocaine	0·05	2	24
		0·1	3	35
	α-Cocaine	0·1	2	20
		0·3	3	30

When the results in Table II were expressed graphically, they showed that 0.6 mg. cocaine was equivalent to 1 mg. α-cocaine, so that α-cocaine was approximately three-fifths as active as cocaine.

TABLE II

COMPARISON OF α-COCAINE WITH COCAINE BY PLEXUS ANAESTHESIA TEST

Substance	Concentration g./100 ml.	Mean Time (min.) to Failure of Response (4 Frogs per Figure)
Cocaine ..	0·05	23
	0·1	18
	0·2	9
α-Cocaine ..	0·1	21
	0·2	14·5

Some observations were also made to see if the quaternized derivative, α-cocaine methiodide, had any local anaesthetic action. By the guinea-pig weal test α-cocaine methiodide was inactive up to 4 g./100 ml., and by the plexus anaesthesia test the activity was very slight and doubtful (up to 1 g./100 ml.).

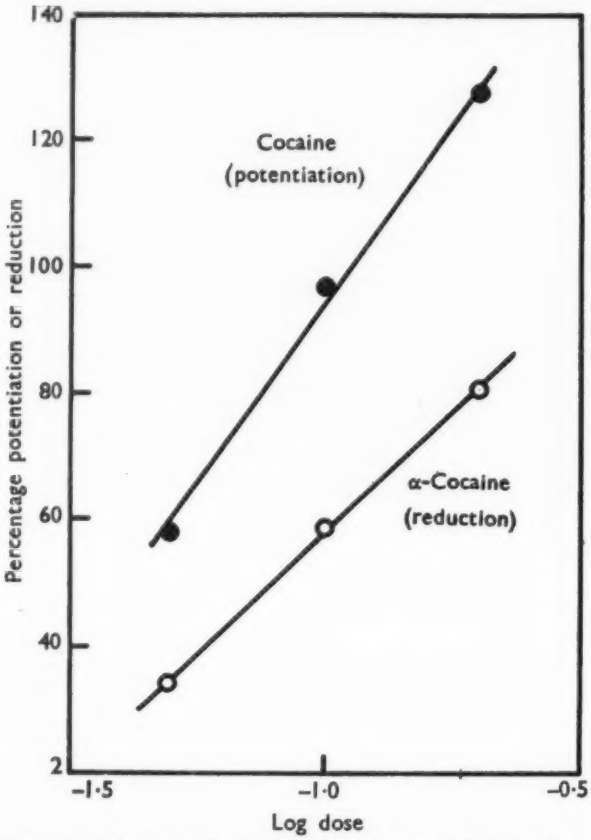

FIG. 2.—Graph of results obtained on the isolated rabbit uterus in a bath of 10 ml. ●——● shows percentage potentiation of effect of adrenaline in relation to log dose cocaine. ○——○ shows percentage reduction of effect of adrenaline in relation to log dose α-cocaine.

Isolated Rabbit Uterus.—Since cocaine is known to potentiate the action of adrenaline in stimulating the isolated rabbit uterus, observations were made to see if α-cocaine had the same action. It was found that α-cocaine had the opposite effect, depressing the action of adrenaline as shown in Fig. 1. Cocaine hydrochloride in concentrations from 5×10^{-3} to 5×10^{-1} mg./ml. potentiated the effects of adrenaline (5×10^{-6} to 4×10^{-5} mg./ml.) in five preparations, and of noradrenaline (6×10^{-5} to 2×10^{-4} mg./ml.). On the other hand, α-cocaine in concentrations from 5×10^{-3} to 8×10^{-1} mg./ml. reduced or abolished the effect of adrenaline in four preparations. In a fifth preparation showing much spontaneous activity, α-cocaine had no effect on the adrenaline response. In one experiment the potentiation produced by cocaine and the reduction of the adrenaline effect by α-cocaine were both in linear relation to the log dose as shown in Fig. 2.

Isolated Rabbit Duodenum.—In the isolated rabbit duodenum cocaine had little or no potentiating effect on the action of adrenaline, but the depression of the action of adrenaline by α-cocaine was still evident. This is illustrated in Fig. 3, and was the result obtained in four preparations. In two preparations cocaine potentiated the action of noradrenaline, whereas α-cocaine diminished it.

Isolated Rabbit Auricles.—When the auricles were suspended in Locke solution at 29° C., the presence of cocaine in a concentration of 2×10^{-3} mg./ml. potentiated the effect of adrenaline. α-Cocaine in the same or double the concentration occasionally potentiated the effect of adrenaline, but less so than cocaine; more frequently potentiation was absent or inhibition occurred. In seven observations potentiation occurred twice, reduction twice and no change thrice.

Spinal Cat: Blood Pressure and Nictitating Membrane.—Experiments were carried out on four spinal cats. As shown in Fig. 4, the effects of adrenaline and of noradrenaline were usually not modified by the intramuscular injection of an amount of α-cocaine as great as 40 mg., but they were always potentiated by the injection of 8 mg. cocaine. The effects on the nictitating membrane were similar as shown in Fig. 4. In one preparation the injection of 80 mg. α-cocaine potentiated

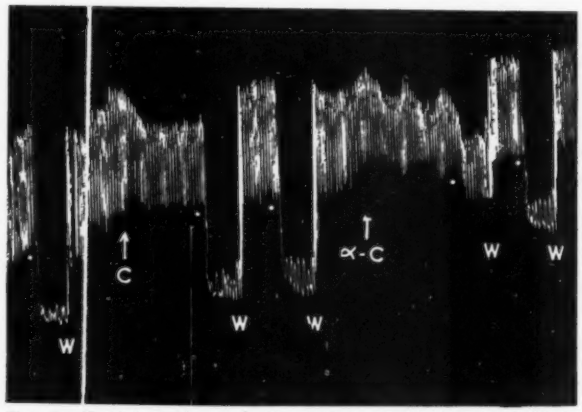

FIG. 3.—Rabbit duodenum in 10 ml. bath. Each white dot indicates the addition of 0·2 μg. adrenaline. W = washout of bath. At C, 0·3 mg. cocaine: at α-C, 0·3 mg. α-cocaine; only the latter depressed the adrenaline response.

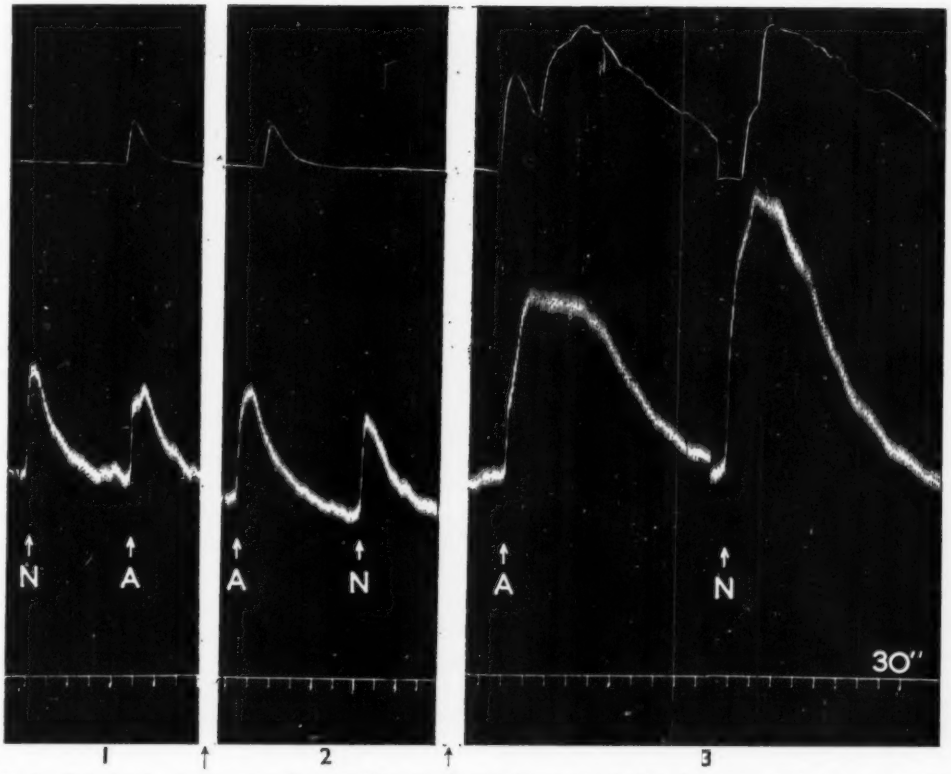

FIG. 4.—Spinal cat. Upper record: nictitating membrane; lower record: blood pressure. N=5 μg. noradrenaline; A=5 μg. adrenaline. Between 1 and 2, 40 mg. α-cocaine; between 2 and 3, 8 mg. cocaine intramuscularly. Note potentiation by cocaine but not by α-cocaine.

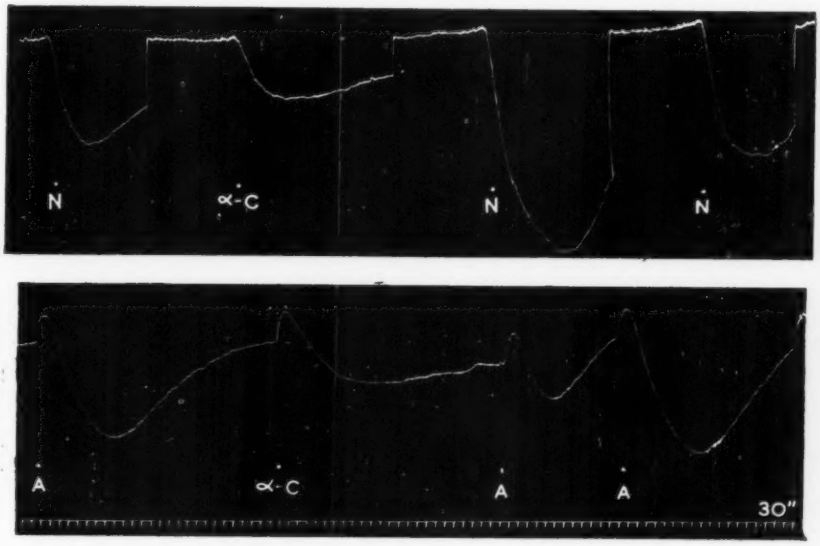

FIG. 5.—Record of outflow from perfused vessels of rabbit ear. Top: 0·001 μg. noradrenaline injected at N, and 0·2 mg. α-cocaine injected at α-C. Note potentiation of constrictor action of N. Bottom (taken from a different experiment): 0·001 μg. adrenaline injected at A, and 0·03 mg. α-cocaine at α-C. Note reduction of constrictor action of A.

the action of adrenaline and of noradrenaline, though much less so than a small dose (8 mg.) of cocaine.

Perfused Rabbit Ears.—Three experiments were carried out in which the isolated rabbit ear was perfused with Locke solution. In one of these the injection of 0.2 mg. α-cocaine potentiated the constrictor action of a subsequent dose of noradrenaline as shown in Fig. 5 (top). In a second experiment the injection of 0.03 mg. α-cocaine diminished the effect of a subsequent dose of adrenaline as shown in Fig. 5 (bottom). In the third experiment, α-cocaine again depressed the action of adrenaline whereas cocaine increased it.

Action of α-Cocaine on Amine Oxidase.—The results obtained for the inhibitory action of α-cocaine on the activity of amine oxidase are shown in Table III. There were seven experiments, three with cat liver and nictitating membrane, and four with rabbit liver and uterus. In the three experiments with cat tissue, the inhibitory action of α-cocaine on amine oxidase was practically identical with that of cocaine; in the four experiments with rabbit tissue the inhibitory action of α-cocaine was rather greater than that of cocaine.

TABLE III

COMPARISON OF INHIBITORY ACTION OF COCAINE AND α-COCAINE ON AMINE OXIDASE

Expt.	Amine Oxidase Preparation	Tyramine Molarity	Cocaine Molarity	α-Cocaine Molarity	Inhibition %
1	Cat liver ..	0·005	0·01	—	40·0
	,, ..	0·005	—	0·01	38·0
2	,, ..	0·005	0·02	—	55·0
	,, ..	0·005	—	0·02	38·0
3	Cat nictitating membrane ..	0·005	0·01	—	39·0
	,, ..	0·005	—	0·01	39·0
4	Rabbit liver	0·005	0·01	—	23 0
	,, ,,	0·005	—	0·01	32·0
5	,, ,,	0·005	0·02	—	44·5
	,, ,,	0·005	—	0·02	55·0
6	,, ,,	0·01	0·02	—	46·0
	,, ,,	0·01	—	0·02	54·0
7	Rabbit uterus	0·005	0·01	—	41·0
	,, ,,	0·005	—	0·01	49·0

The following solutions were used: enzyme, 1·0 ml.; tyramine, 0·05M, 0·2 and 0·4 ml.; cocaine and α-cocaine, 0·1M, 0·2 and 0.4 ml.; KCN, 0·01M, 0·2 ml.; KOH/KCN (centre well), 0·2 ml.; phosphate buffer, 0·067M, *p*H 7.4, to bring to final volume of 2·2 ml. The rate was measured as the extra oxygen uptake over that of the control during the period 0–20 min., except for Expt. 3.

DISCUSSION

α-Cocaine has been found to have a local anaesthetic action when injected into the skin of the guinea-pig, and also when allowed to act on the nerves entering and leaving the spinal cord of the frog in the lumbar region. The action in both tests was found to be weaker than that of cocaine. The results on the rabbit cornea were ambiguous; in nine of thirteen rabbits α-cocaine (5%) was found to have no local anaesthetic action, but in one rabbit a 5% solution was active and in three rabbits a 1% solution was active. These results indicate that α-cocaine is not a reliable surface anaesthetic and probably explain Willstätter's failure to observe anaesthesia of the tongue.

Since the original observation of Fröhlich and Loewi (1910) the potentiation of the action of adrenaline by cocaine has been repeatedly confirmed. In 1926 Tainter and Chang showed that cocaine acted differently on tyramine, reducing or abolishing its pressor effect. Tainter (1930) also showed that on the blood pressure of the spinal cat the potentiation of adrenaline became steadily less with doses of cocaine higher than 10–15 mg. per kg., and in the same paper recorded that psicaine, benzoylpseudotropeine and β-eucaine were unable to potentiate the action of adrenaline. He found further that these substances were unable to reduce the pressor response of tyramine. Hence substances closely similar in constitution to cocaine lacked its potentiating or depressant actions on sympathomimetic amines.

An effect of α-cocaine on the adrenaline response was found present in isolated organs; thus on the auricles of the rabbit potentiation, reduction and no alteration of the action of adrenaline were all observed. When potentiation occurred it was less than that produced by the same concentration of cocaine. On the isolated uterus of the rabbit α-cocaine diminished the action of adrenaline whereas cocaine increased it. This difference is not necessarily a difference in kind from the action of cocaine; Burn and Tainter (1931) observed that cocaine diminished the action of adrenaline in inhibiting the isolated uterus of the cat. On the small intestine of the rabbit α-cocaine depressed the action of adrenaline, cocaine having little or no effect. On the blood pressure of three spinal cats, α-cocaine did not modify the pressor action of either adrenaline or noradrenaline in amounts up to 40 mg., but in one cat the effects of adrenaline and noradrenaline were potentiated after the injection of 80 mg. In the vessels of the rabbit ear, small doses of α-cocaine (0.03 mg.) depressed the action of adrenaline in two experiments; in one experiment a large dose (0.2 mg.) potentiated the action of noradrenaline.

Since Philpot (1940) showed that cocaine exerted an inhibitory action on amine oxidase, tests were made with α-cocaine and cocaine in parallel. With tyramine as substrate, α-cocaine

was found to have the same inhibitory action as cocaine on amine oxidase from cat liver and nictitating membrane, and rather more inhibitory action than cocaine on amine oxidase from rabbit liver and rabbit uterus. The suggestion of Burn and Robinson (1952) that the potentiation of noradrenaline and of adrenaline by cocaine might be due to inhibition of amine oxidase seems therefore to be incorrect.

Summary

1. α-Cocaine was found to possess local anaesthetic activity when examined by intradermal injection into guinea-pigs or by application to the lumbar nerve plexus in frogs. By the former test it was one-fifth as active as cocaine, by the latter it was three-fifths as active. α-Cocaine was without appreciable action on the cornea of the rabbit eye.

2. In isolated preparations of the rabbit α-cocaine occasionally potentiated the action of adrenaline on the auricles, though it was less active than cocaine; more frequently reduction or no alteration of the adrenaline response was observed. It depressed the action of adrenaline on the uterus, intestine, and perfused-ear preparations.

3. In the spinal cat α-cocaine did not modify the actions of adrenaline or noradrenaline on the blood pressure and the nictitating membrane in doses five times larger than potentiating doses of cocaine.

4. α-Cocaine and cocaine had similar inhibitory effects on preparations of amine oxidase from the cat and the rabbit.

This work was done while one of us (V. V.) held a World Health Organization Fellowship. Our thanks are due to Mrs. F. J. Philpot for the observations on amine oxidase, and to Miss Roneen Hobbs for the estimations of local anaesthetic activity.

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THE ENZYMIC HYDROLYSIS OF COCAINE AND *ALPHA*-COCAINE

BY

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Some time ago Glick and Glaubach (1941) reported that cocaine is hydrolysed by rabbit serum ; other mammalian species do not hydrolyse cocaine. In earlier work from this laboratory (Blaschko, Chou and Wajda, 1946) it was confirmed that cocaine is hydrolysed also in the blood of rabbits which do not contain the tropinesterase described by Glick and Glaubach.

α-Cocaine has recently become available (Foster, Ing, and Varagić, 1955), and we have taken the opportunity of examining α-cocaine as a substrate of mammalian esterases. A few experiments on the hydrolysis of benzamine (β-eucaine) are also reported.

METHODS

Human plasma was obtained by centrifugation of a sample of citrated blood stored for blood transfusions. Horse serum was a sample that came from the Lister Institute ; ox and sheep serum was from blood obtained from the slaughterhouse. Most of the experiments with rabbit serum were carried out with the pooled sera of a number of tropinesterase-negative animals.

In the manometric experiments the total reaction volume in each manometer flask was 3.0 ml. The main compartment of the flask contained the enzyme sample and any inhibitors that were used ; the volume was brought up to 2.7 ml. with Krebs bicarbonate-Ringer. The side bulb contained the substrate, in a volume of 0.3 ml. The temperature of the thermostat was 37.5° C., and the gas phase was a mixture of 95% N_2 and 5% CO_2. In most experiments a "substrate blank" was included, in order to correct for the slow, spontaneous hydrolysis of the substrates.

RESULTS

Hydrolysis of α-Cocaine in Different Species.—It was easy to demonstrate that α-cocaine was hydrolysed by rabbit serum. This hydrolysis, like the hydrolysis of cocaine, was seen with sera of both tropinesterase-positive and tropinesterase-negative animals. The rate of hydrolysis of α-cocaine exceeded that of cocaine, but differed a little in different experiments. We have not determined the saturation concentration of the rabbit esterase for α-cocaine, but there was no difference in the initial rates with a concentration of 10^{-3}M- and 10^{-2}M-α-cocaine ; later the rates of hydrolysis fell off, and this fall was more marked with the lower concentration.

Horse serum hydrolysed α-cocaine, whereas cocaine was not attacked. The rate of hydrolysis with horse serum was of the same order of magnitude as with rabbit serum, and there was a similar slow falling off of the rate in an experiment with an initial α-cocaine concentration of 5×10^{-3}M.

Human plasma also hydrolysed α-cocaine, but it was without action on cocaine. Here, too, there was a slow decrease in the rate of hydrolysis with time.

With ox serum, there was only a very small increase in the rate of hydrolysis over that in the manometer flask which served as "substrate blank."

The Action of Eserine.—The hydrolysis of α-cocaine by horse serum and by human plasma was inhibited by eserine. This is seen in Fig. 1, which shows the rates of liberation of CO_2 with different eserine concentrations: 1a is from an experiment with horse serum ; 1b from one with human plasma. It can be seen that with higher concentrations of eserine the inhibition of α-cocaine hydrolysis by human plasma was almost complete. Almost 20% of the activity of horse serum was left with 10^{-4}M-eserine ; with 10^{-5}M- and 10^{-6}M-eserine the rates of hydrolysis were practically identical.

The Action of Nu 683.—This substance, the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide, was first used by Hawkins and Gunter (1946) as a specific inhibitor of pseudo-cholinesterase. We have found that Nu 683 is also an inhibitor of the enzymic hydrolysis of α-cocaine by horse serum and by human plasma. This is illustrated by experiments with

5×10^{-3}M-α-cocaine as substrate, in which different concentrations of Nu 683 were used. The percentage inhibitions in the first 12 min. were :

Concentration of Nu 683	With horse serum	With human plasma
10^{-4}M	85%	100%
10^{-6}M	81 ,,	92 ,,
10^{-8}M	44 ,,	34 ,,

In another similar experiment with horse serum, the percentage inhibitions were:

with 10^{-4}M-Nu 683 ..	79%
,, 10^{-5}M ,, ,, ..	78 ,,
,, 10^{-6}M ,, ,, ..	77 ,,
,, 10^{-7}M ,, ,, ..	76 ,,

These experiments show that, as with eserine, most of the enzymic activity was inhibited, but that with horse serum there remained an activity which was relatively resistant to Nu 683.

Nu 683 (10^{-6}M) did not inhibit the hydrolysis of α-cocaine by rabbit serum.

The hydrolysis of benzoylcholine, a typical substrate of pseudo-cholinesterase, was inhibited by Nu 683.

Mixed-substrate Experiments.—In an experiment with rabbit serum, in which both α-cocaine and cocaine were used as substrates, the rate of hydrolysis with the two compounds present simultaneously was intermediate between the rate with either cocaine or α-cocaine alone. In the first 15 min. the amounts of CO_2 liberated were:

With cocaine 2×10^{-2}M:	16 μl. CO_2
,, α-cocaine 2×10^{-2}M:	39 ,, ,,
,, both substrates:	27 ,, ,,

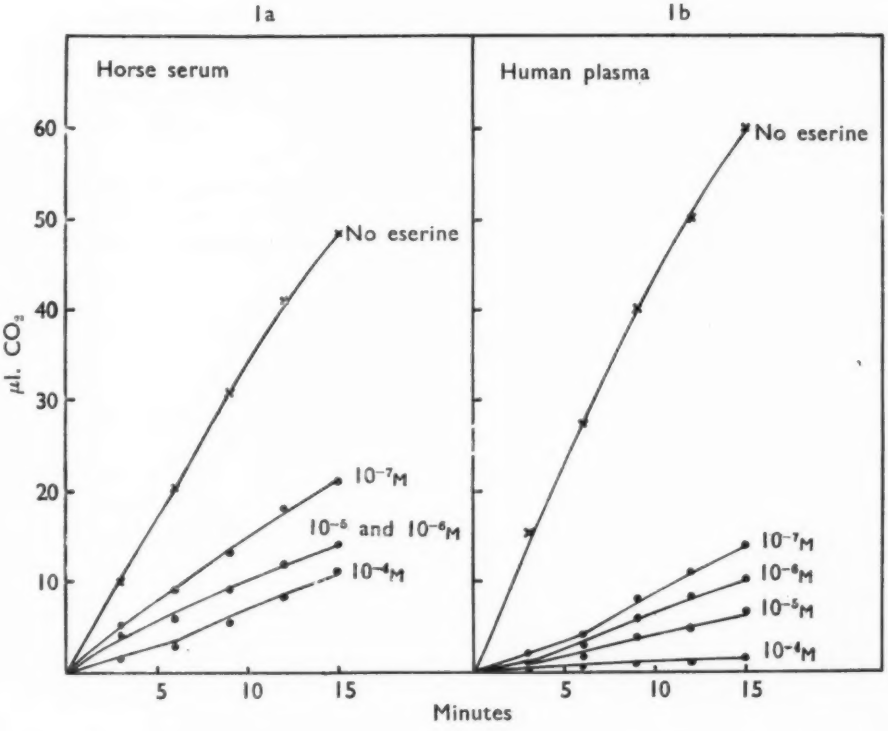

FIG. 1.—Inhibition of enzymic hydrolysis of α-cocaine by eserine. 1a: horse serum. 1b: human plasma. Abscissa, time in min. Ordinate, μl. CO_2 consumed. Initial concentration of α-cocaine: 0·005M. The eserine concentrations used are shown on the graphs.

In other words, the rate of the reaction in the mixed-substrate experiment was competitive, not additive.

A mixed-substrate experiment was also performed with horse serum; benzoylcholine and α-cocaine were the two substrates. The CO_2 liberated in the first 15 min. was:

With α-cocaine 5×10^{-3}M:	12 μl. CO_2
,, benzoylcholine 6×10^{-3}M:	51 ,, ,,
,, both substrates:	24 ,, ,,

In this experiment 0.2 ml. horse serum was used in each manometer flask.

Experiments with Benzamine (4-Benzoyloxy-2:2:6-trimethyl-piperidine).—This compound was readily hydrolysed by both human plasma and horse serum; it was less rapidly hydrolysed by rabbit serum, and with ox and sheep serum there was no hydrolysis.

The hydrolysis of benzamine was inhibited by Nu 683; this is shown in Table I.

TABLE I

INHIBITION OF HYDROLYSIS OF BENZAMINE (β-EUCAINE) BY NU 683

The percentage inhibitions are based on the hydrolysis during the 3–12 min. interval after tipping.

Source of Enzyme	Concentration of Nu 683		
	10^{-4}M	10^{-6}M	10^{-8}M
Rabbit serum	53%	13%	16%
Human plasma	98 ,,	89 ,,	48 ,,
Horse serum	100 ,,	96 ,,	51 ,,

DISCUSSION

As a result of these experiments it seems likely that in man and horse α-cocaine is inactivated by enzymic hydrolysis, whereas cocaine is not hydrolysed in human and horse plasma. In the rabbit, no similar difference between cocaine and α-cocaine was seen; both compounds were hydrolysed, α-cocaine somewhat faster than cocaine.

It seems quite possible that the catalyst of the hydrolysis of α-cocaine in man and horse is pseudo-cholinesterase. The hydrolysis was not only inhibited by eserine, but also by the rather more specific inhibitor, Nu 683; moreover, an inhibition of the hydrolysis of α-cocaine is obtained with concentrations of Nu 683 similar to those which inhibit the hydrolysis of benzoylcholine (Blaschko, Bülbring and Chou, 1949). Also in favour of the identity of the α-cocaine esterase with pseudo-cholinesterase are: first, the slow rate of hydrolysis with ox serum, a preparation that has no pseudo-cholinesterase activity; and second, the competitive rates of hydrolysis of α-cocaine and benzoylcholine in the mixed-substrate experiments. There is the possibility that in horse serum a second enzyme is responsible for the small eserine- (and Nu 683-) resistant hydrolysis of α-cocaine. Little, if any, of this second enzyme was present in the sample of human plasma used in our experiments.

The catalyst of the hydrolysis of α-cocaine in rabbit serum must be different from pseudo-cholinesterase. Firstly, there was no inhibition of α-cocaine hydrolysis by Nu 683, whereas the slow hydrolysis of benzoylcholine was inhibited, and, secondly, the rate of hydrolysis of a mixture of cocaine and α-cocaine was intermediate between the rates with either substrate alone. This latter observation suggests the possibility that in the rabbit the two esters are hydrolysed by one and the same esterase.

It is interesting that the hydrolysis of the other ester tested, benzamine, has a distribution which is similar to the distribution of pseudo-cholinesterase: the compound was rapidly hydrolysed by human plasma and horse serum, two preparations known to be rich in this type of cholinesterase; it was slowly hydrolysed by rabbit serum which is poor in this enzyme, and ox and sheep sera did not act on benzamine; it is known that ruminants do not contain pseudo-cholinesterase.

Observations like those reported in this study are of interest, as the time course of action of these drugs will depend on whether or not they are inactivated by hydrolysis. It can be expected from our findings that the relative potencies will depend upon the species used for biological assays.

SUMMARY

1. The enzymic hydrolysis of α-cocaine in mammalian plasma has been studied.

2. Horse serum and human plasma hydrolysed α-cocaine rapidly; the hydrolysis in horse serum was partly, that in human serum completely, inhibited by inhibitors of pseudo-cholinesterase.

3. Both α-cocaine and cocaine were hydrolysed by rabbit serum; the evidence suggests that the same enzyme is catalysing these reactions and that it is distinct from pseudo-cholinesterase.

4. Benzamine (β-eucaine) was hydrolysed rapidly by human plasma and horse serum, less readily by rabbit serum; it was not hydrolysed by ox or sheep serum.

We are grateful to Drs. R. Foster and H. R. Ing for the sample of α-cocaine hydrochloride, and to Messrs. Parke, Davis & Co. for the benzamine hydrochloride.

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THE ACTION OF 5-HYDROXYTRYPTAMINE ON THE BLOOD VESSELS OF THE HUMAN HAND AND FOREARM

BY

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Rapport, Green, and Page in 1948 isolated a vasoactive substance ("serotonin") from beef serum and this was later shown (Rapport, 1949) to be 5-hydroxytryptamine, the creatinine sulphate (5-HT) of which has since been produced synthetically (Hamlin and Fischer, 1951 ; Specter, Heinzelmann, and Weisblat, 1951). The presence of 5-HT has been demonstrated in many body tissues and fluids of man and animals, including the alimentary canal (Dalgliesh, Toh, and Work, 1953), brain and urine (Twarog and Page, 1953), enterochromaffin tissue (Erspamer and Asero, 1952), blood serum (Erspamer and Sala, 1954) and platelets (Zucker and Borelli, 1955). It has been suggested that this substance plays a part in haemostasis (Zucker, 1947), in kidney function (Erspamer and Sala, 1954) and in central nervous activity (Stole, 1947). Its effects on the circulation suggest that it has a role in the regulation of vascular tone (Page, 1952). The mechanism of this last effect is still obscure and the present paper attempts to define the actions of 5-HT on peripheral blood vessels in man by observation of blood flow, heat loss and volume changes in the upper limb during local arterial injections.

METHODS

Experiments were carried out on 8 healthy subjects (7 men and 1 woman) of ages 18–32 years. The subject lay recumbent on a couch for half to three-quarters of an hour before the beginning of the experiment. The room temperature was 20–24° C. in different experiments and did not vary by more than 1° C. in any one experiment. An infusion of 0.9% saline was started at a rate of 4 ml./min. through a needle inserted into the left brachial artery at the elbow and connected by a short length of polythene tubing to one of two mechanically driven syringes. These syringes could be driven simultaneously and the infusion changed from saline to drug solution without delay.

For each experiment 2–5 mg. of 5-hydroxytryptamine creatinine sulphate salt (Abbott) was dissolved in 5 ml. of saline in a sealed glass ampoule and boiled for 15–20 min. This solution was then diluted so that the dose for 1 min. was contained in 4 ml. of saline. This treatment has been shown not to affect the potency of 5-HT as determined by its action on the guinea-pig ileum (Mongar, personal communication). Infusions were given into the brachial artery of one side in doses ranging from 0.25 to 16 μg./min. for periods of 5–15 min. All doses are expressed as weight of the creatinine sulphate salt.

Observations were made on both limbs simultaneously, the right being used as a control throughout. Measurements of blood flow through the forearms or hands were made by venous occlusion plethysmography at 35 and 32° C. respectively using the temperature-controlled, stirred plethysmographs described by Greenfield (1954). Volume changes in the hands and forearms were also measured with the plethysmographs. In some experiments heat elimination from the pulps of the middle fingers of the two sides was measured with copper-tellurium heat-flow discs (Hatfield, 1950) simultaneously with measurements of blood flow or volume change.

RESULTS

Subjective Effects

Soon after beginning the infusion of 5-HT in doses of 1 μg./min. and above, a sensation of tingling was felt in the hand and lower forearm which decreased somewhat throughout the infusion period and subsided within a few minutes of the end of the infusion. No generalized effects were noted.

Hand Circulation

In three subjects the hand blood flow was measured plethysmographically during infusions of 0.25 to 16 μg./min. (Fig. 1). Doses of 1 μg./min. or less had no effect on the flow; doses of 4 and 16 μg./min. reduced the flow, 16 μg. appearing to arrest the circulation almost completely. During the infusion of the drug the hand volume increased and when the wrist cuff was inflated to impede the venous return the additional amount of blood which could be accommodated in the hand was less than before the infusion, and hence the inter-

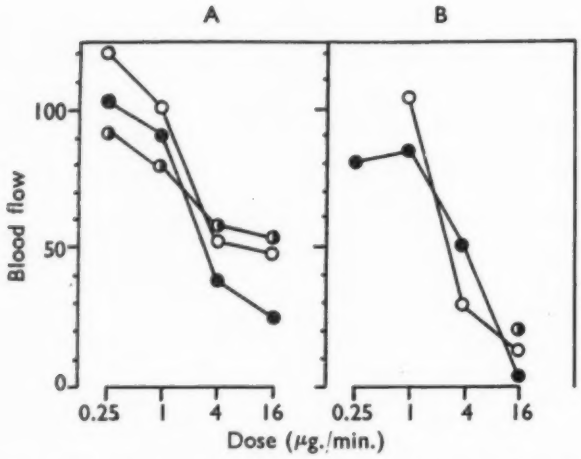


FIG. 1.—Dose-response curves for 5-hydroxytryptamine on forearm (A) and on hand (B) blood flow. The flow (ordinates) is expressed as a percentage of the predicted value if no infusion had been given, determined from the ratios of the resting levels of flow in the control and experimental sides. (See Greenfield and Patterson, 1954.)

pretation of the inflow curves was rendered somewhat uncertain. Typical examples of hand blood-flow records before and during 5-HT infusion are shown in Fig. 2.

In one of the above experiments the heat flow was recorded from the pulps of the middle fingers simultaneously with the blood flow measurements and the changes in heat elimination confirmed the interpretation of the plethysmograph findings (Fig. 3). The reduction in hand circulation during 5-HT infusions was seen in 2 further experiments in

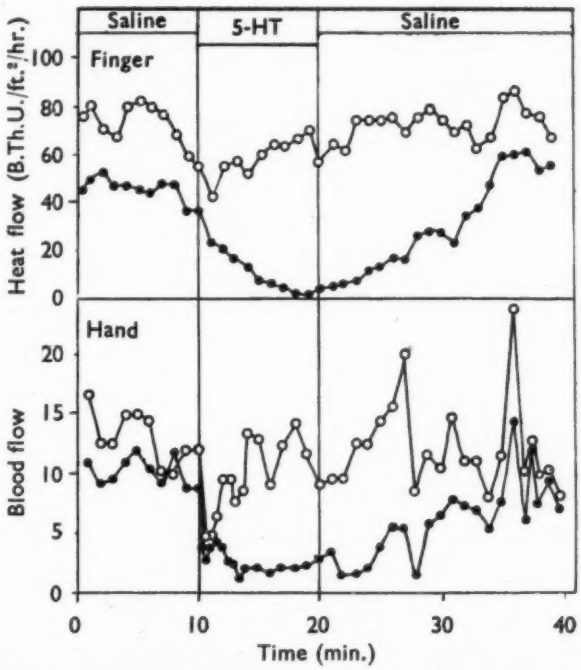


FIG. 3.—Simultaneous hand blood flow and finger heat flow measurements before, during, and after infusion of 16 μg./min. 5-hydroxytryptamine into the brachial artery of the left side. ● Left (injected) side; ○ right (control) side. Blood flow in ml./100 ml./min.

which the heat elimination was recorded simultaneously with changes in volume of the hands (Figs. 4 and 5A). Concurrent with the fall in heat elimination the hand volume increased.

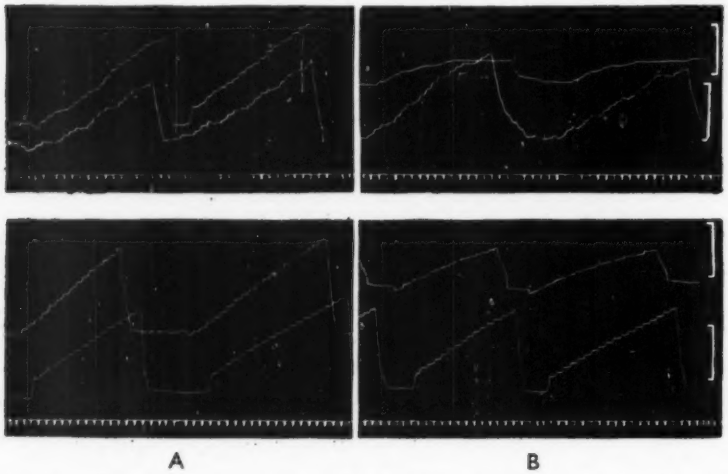


FIG. 2.—Typical plethysmographic records of hand blood flow (upper frames) and forearm blood flow (lower frames). A, 1 min. before, and B, 8 min. after the beginning of infusions of 5-hydroxytryptamine (4 μg./min.). The upper record in each frame was obtained from the experimental limb and the lower record from the opposite (control) limb. The calibration marks represent 5 ml. increases in volume. Time, 1 sec.

In Fig. 6 the rate of fall in heat elimination from the finger pulp in 3 subjects following the onset of infusions of 5-HT at 16 μg./min. is plotted logarithmically against time and compared with the rate of fall in a subject in whom the circulation to the arm was arrested by inflation of a pneumatic cuff around the upper arm to 260 mm. Hg. The results demonstrate that the infusion arrested the circulation through the fingers completely or almost completely.

With doses of 16 μg./min. there was an initial transient vasoconstriction in the control hand (Figs. 3 and 4). No such effect was seen in the control forearm (Figs. 5B and 7), and since the constriction was transient and coincided with the onset of the sensation of tingling it was probably reflex

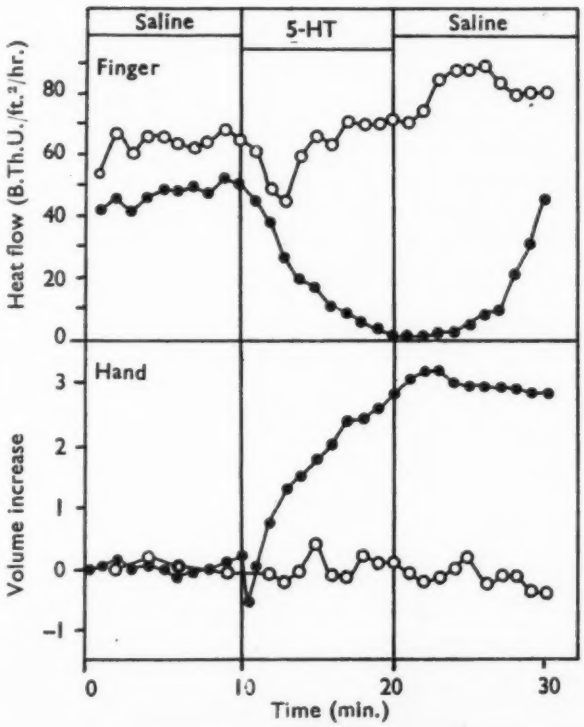


FIG. 4

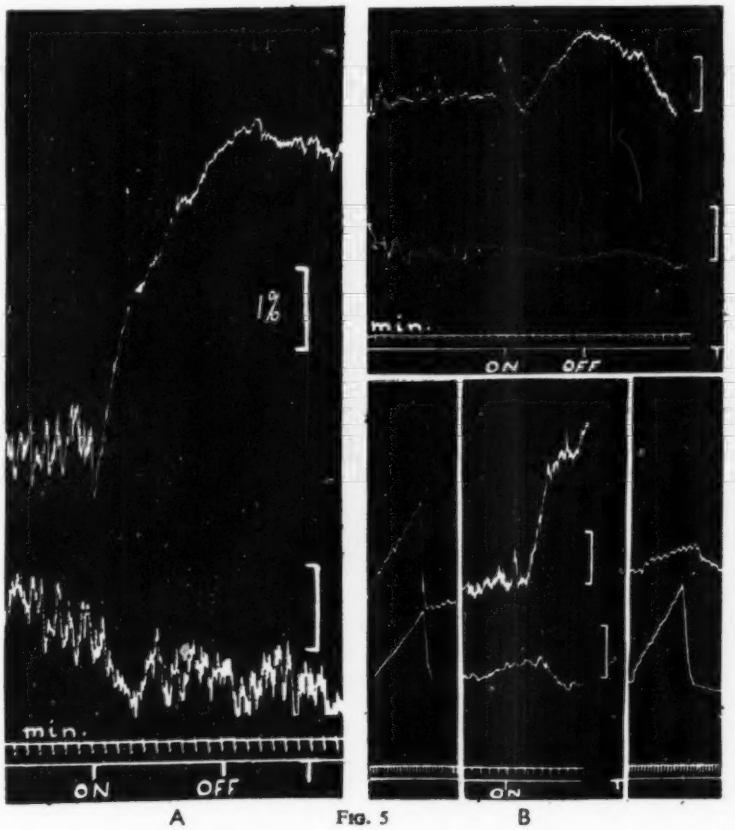
in origin and not due to the arrival of 5-HT in the control limb.

Observations of colour changes in the hands and fingers were made in 2 subjects during infusion of 5-HT. A bright red flush of the skin of the hand

FIG. 4.—Simultaneous finger heat flow and hand volume change during infusion of 16 μg./min. of 5-hydroxytryptamine into the left brachial artery. ● Left (injected) side; ○ right (control) side. Volume changes are expressed as percentage increase from the resting volume (465 ml.).

FIG. 5.—Records of volume changes during infusions of 5-hydroxytryptamine into the left brachial artery. A, *Hand*, during infusion of 16 μg./min. B, *Forearm*. *Upper record*, during infusion of 1 μg./min. *Lower record*, left-hand frame, inflow curves before infusion; centre frame, forearm volume change during infusion of 16 μg./min.; and right-hand frame, inflow curves 8 min. after the start of the infusion. The calibration marks represent a change in volume of 1% and also indicate the static calibration. The lower tracing in each record is from the control limb.

FIG. 6.—Comparison of the rate of fall in heat elimination (log scale) from a finger of each of 3 subjects immediately following the start, at time 0, of an infusion of 16 μg./min. 5-hydroxytryptamine into the brachial artery (circles) and the rate of fall following arrest, at time 0, of the circulation by a pneumatic cuff around the upper arm inflated to 260 mm. Hg (dots).



A FIG. 5 B

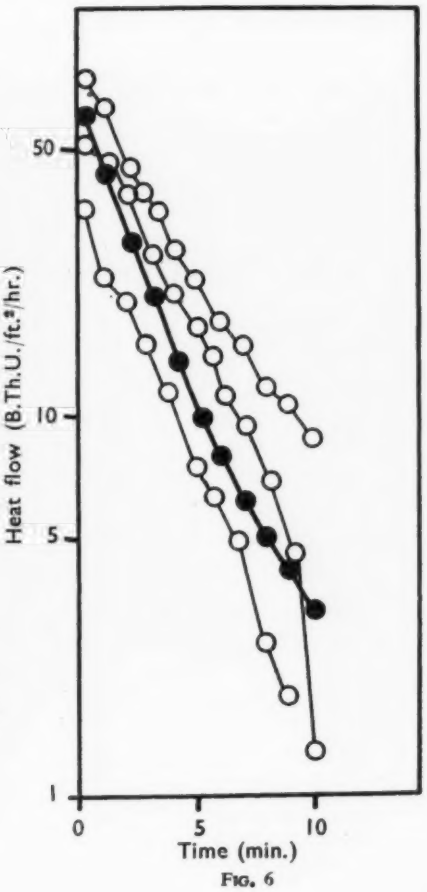


FIG. 6

and fingers appeared within 1–2 min. and was followed after about 5–7 min. by a dusky blue discoloration of the fingers which increased during the remainder of the 10 min. infusion so that by the end of this period the fingers were deeply cyanotic. This appearance subsided during the subsequent 10 min., the hand and fingers returning to their normal colour.

Forearm Circulation

Fig. 5B illustrates the increases in forearm volume which occurred during infusion of 1 and 16 μg./min. of 5-HT into the brachial artery. With 16 μg. the increase was of the order 2–3%. In Figs. 2 and 5B (lower frame) are shown records obtained by venous occlusion plethysmography before and during infusions of 4 and 16 μg./min. at a time when the changes in volume were maximal. If it is valid in view of the increase in forearm volume to assess changes in the rate of arterial inflow from these records at least qualitatively, then it is clear that the drug causes a reduction in forearm flow.

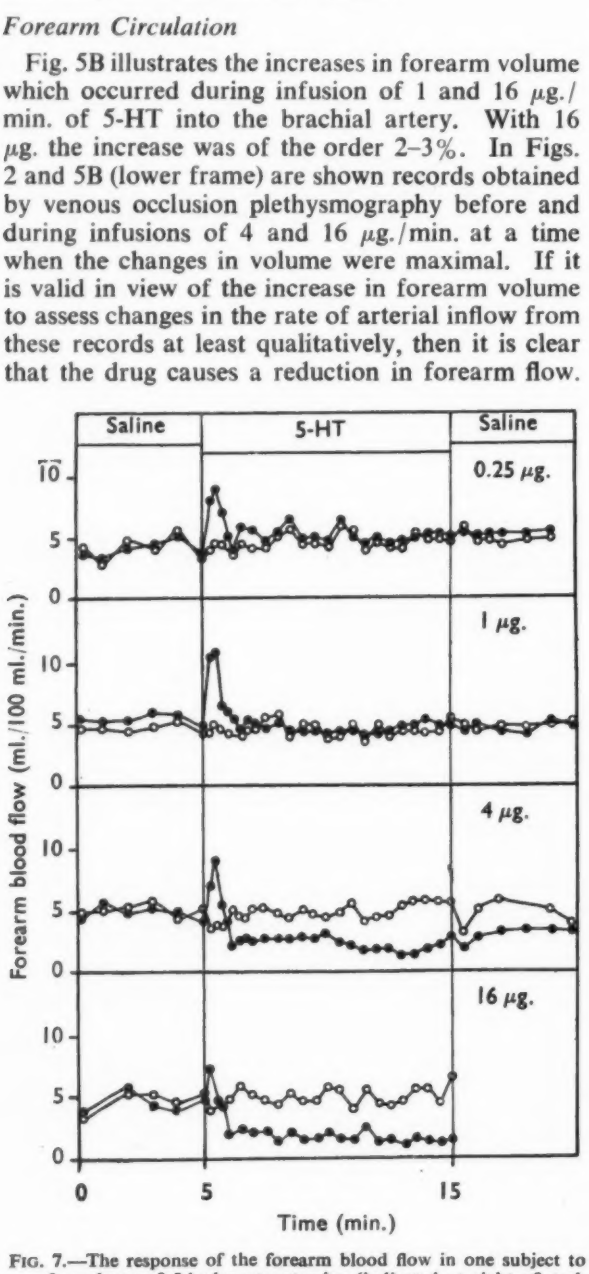


FIG. 7.—The response of the forearm blood flow in one subject to four doses of 5-hydroxytryptamine (indicated at right of each frame) infused into the brachial artery of the left side. ● Left forearm; ○ right forearm.

Fig. 7 illustrates the effects of various doses on the forearm blood flow on the assumption that the plethysmographic records are indicative of the true rates of arterial inflow. At all dose levels the forearm flow was briefly increased during the first 60–90 sec. of the infusion. At dose levels of 1 μg./min. or less the flow during the remainder of the infusion did not differ from that of the control side. At 4 and 16 μg./min., following the initial transient increase, the forearm flow fell to half to one-third of the resting value and remained so for the rest of the infusion. Fig. 1 shows the dose-response curve for the forearm flow during the last half of the infusion period in 3 subjects.

The colour changes in the forearm were as striking as those in the hand. Infusion of doses of 4 μg. or more caused a diffuse flush of the forearm skin, particularly on the flexor surface; this persisted throughout the infusion and gradually subsided afterwards. At no time was the skin of the forearm cyanosed.

In one subject, who received an intra-arterial infusion at a rate of 4 μg./min. for 8 min., and in 3 who received 16 μg./min. for 10–15 min., there developed, after a latent period of some hours, small petechial haemorrhages either scattered diffusely over the flexor surface of the forearm or grouped on the flexor surface of the wrist and lower forearm. These were associated with a slight itching and faded in 24–48 hr.

DISCUSSION

The increase in forearm and hand volume during 5-HT infusions might render invalid the assessment of the rate of arterial inflow by the plethysmographic method, especially as it seems most probable that the increase is due to dilatation of the capacity vessels. That the inflow curves do at least reflect the direction of change of flow, however, is demonstrated by the fact that, in the hand, the decrease in slope of the curves is closely paralleled by a decrease in heat elimination. That the blood flow through the skin of the hand is very much reduced in spite of dilated capillaries is further indicated by the development of cyanosis of the skin of the fingers during the infusion. In the forearm the apparent reduction in flow obtained by the plethysmographic method could not be checked by calorimetric or skin temperature measurements, since these cannot be used to assess changes in forearm circulation with any degree of accuracy. The effect of 5-HT on the forearm flow could, therefore, not be interpreted with the same certainty as in the hand.

The increase in forearm and hand volume during infusion of the drug must be contributed to—at least in part—by the dilatation of the minute vessels of the skin. It is possible that other factors such as venoconstriction (Reid, 1952) or oedema formation may contribute, but the present experiments provide no evidence on these points. The development of petechial haemorrhages following the larger doses in some subjects is indicative of capillary damage. Whether some of the effects of 5-HT on the peripheral vessels might be accounted for by release of histamine is still uncertain. Feldberg and Smith (1953) have shown that large doses of 5-HT cause release of histamine from the perfused skin flap of the cat, accompanied by vasoconstriction and oedema formation. Although histamine, in a wide range of doses, injected intra-arterially into the forearm or hand in man, always causes dilatation of the resistance vessels and an increase in flow (Duff, Greenfield, Shepherd, and Thompson, 1953), it is conceivable that the direct constrictor action of 5-HT on the resistance vessels might counteract the dilator action of histamine on these vessels while leaving unopposed its action on the capillaries. On the other hand, Reid (1952) demonstrated that the flush of the skin caused by intradermal injection of 5-HT was not accompanied by itching or a weal, and preliminary experiments in this department indicate that there is no diminution of the flush following intra-arterial injection of antihistamine.

The effect of intra-arterial administration of 5-HT in doses of more than 1 μg./min. on the circulation in the hand and forearm in man is essentially the same. The blood flow is very considerably reduced, while there is an increase in the volume of the part and a flushing of the skin. In the forearm a transient vasodilatation precedes the vasoconstriction and with doses of less than 1 μg./min. the transient dilatation alone is observed. This dilatation did not increase with increasing doses.

These results demonstrate that intra-arterial 5-HT constricts those vessels which control the rate of blood flow (the arterioles) and at the same time dilates the minute vessels of the skin.

In recent years a clinical and pathological triad has been described (Thorson, Biörck, Björkman, and Waldenström, 1954 ; Isler and Hedinger, 1953 ; Rosenbaum, Santer, and Claudon, 1953 ; and Branwood and Bain, 1954) consisting of carcinoid tumour, pulmonary stenosis and a reddish-blue colour of the skin often associated with periodic attacks of intense flushing. Pernow and Waldenström (1954) have demonstrated an increase in substances resembling 5-HT in the blood and urine of 2 patients with carcinoid tumours during attacks of flushing. They have suggested that the vasomotor symptoms are caused by a discharge of 5-HT. The skin colour changes observed in the present experiments are consistent with this view.

Summary

1. The circulation in the upper limb has been observed during infusions of 5-hydroxytryptamine creatinine sulphate (5-HT) in doses of 0.25 to 16 μg./min. into the human brachial artery.

2. With doses of more than 1 μg./min. the forearm and hand blood flow decreases, the volume increases and there is a marked flushing of the skin.

3. 5-HT causes a constriction of the vessels mainly responsible for resistance to flow, and a dilatation of the vessels responsible for the colour of the skin.

4. In the forearm the fall in flow is preceded by a transient vasodilatation.

5. As the infusion continues, cyanosis gradually develops in the skin of the fingers but not elsewhere.

6. In some subjects small petechial haemorrhages were observed some hours after the infusion.

7. The colour changes in the skin are similar to those that have been described in some cases of carcinoid tumour of the intestine.

We wish to express our thanks to those students and colleagues who acted as subjects, to Professor A. D. M. Greenfield for advice and criticism in the preparation of the paper, and to Abbott Laboratories, Chicago, for a supply of 5-hydroxytryptamine.

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THE INHIBITION OF AMINE OXIDASE AND SPERMINE OXIDASE BY AMIDINES

BY

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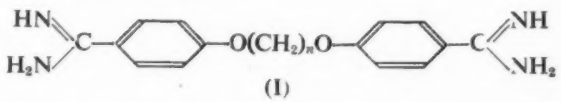
It has been known for some time that the homologous series (I)



(I)

includes substances which are strong inhibitors of the amine oxidase of rabbit liver (Blaschko and Duthie, 1945). The two most active members of the series are propamidine ($n=3$) and pentamidine ($n=5$).

While experimenting with a preparation of the enzyme from guinea-pig liver, it was recently noted that this enzyme was much less strongly inhibited by pentamidine. We have therefore examined all the available members of the homologous series with preparations of amine oxidase from rabbit, guinea-pig, dog, cat, sheep, pig, ox, squid and sea-urchin. The two invertebrates were chosen as representatives of phyla in which amine oxidase is present. The inhibitory action of the amidines upon the spermine oxidase of sheep and ox serum was also studied.

METHODS

Most of the amidines tested were the original samples obtained in 1945 from Dr. A. J. Ewins, F.R.S.; in addition, the octamethylene derivative as the diisethionate, was made available through the kindness of Dr. R. Wien, of Messrs. May and Baker.

The source of mammalian amine oxidase was liver; we also used pig kidney in order to compare enzyme from two different tissues in the same species.

The amine oxidase preparations were fresh extracts, dialysed against tap water, with sodium phosphate buffer of *p*H 7.4 added in the manometer flask. The livers of the squid (*Loligo forbesii*) and the gonads of the sea-urchin (*Echinus esculentus*) were from animals dissected in Plymouth six months earlier and kept frozen at $-10°$. All the extracts were made up before dialysis, so that 1 g. of wet tissue was present in 2 ml. In order to obtain suitable manometer readings, different volumes of the tissue preparations were used in different experiments: they varied from 0.15 ml. in one of the experiments with ox liver to 1.4 ml. with sea-urchin gonads. Oxygen uptakes in the first 15 min. differed from 20.5 μl. in one experiment with squid liver to 135 μl. in one experiment with pig kidney.

The spermine oxidase preparations were ox or sheep serum, dialysed against 0.067M-sodium phosphate buffer of *p*H 7.4.

In the manometric experiments, the main compartment of the manometer flasks contained the enzyme preparation, brought to a total volume of 1.6 ml. by adding sodium phosphate buffer; the side arm contained 0.2 ml. of substrate (0.1M-tyramine hydrochloride or 0.05M-spermine tetrahydrochloride) plus 0.2 ml. of either water or inhibitor. An " enzyme blank " was also included. The gas phase was oxygen and the temperature was 37.5°.

For the calculation of the percentage inhibitions, the 0 to 15 min. readings were used when tyramine was the substrate; in the experiments with spermine the calculation was based on the 5 to 25 min. readings. During these periods the reaction rates remained approximately linear.

RESULTS

The results are shown graphically in the twelve diagrams of Fig. 1. Each of the diagrams shows the source of enzyme, the molar concentrations of the inhibitors used, and percentage inhibitions obtained. Each point represents one result.

The results, as shown in the diagrams, may be summarized as follows:

(*a*) The inhibition of amine oxidase by the amidines tested differs from species to species. Thus, for example, the amine oxidase of the rabbit

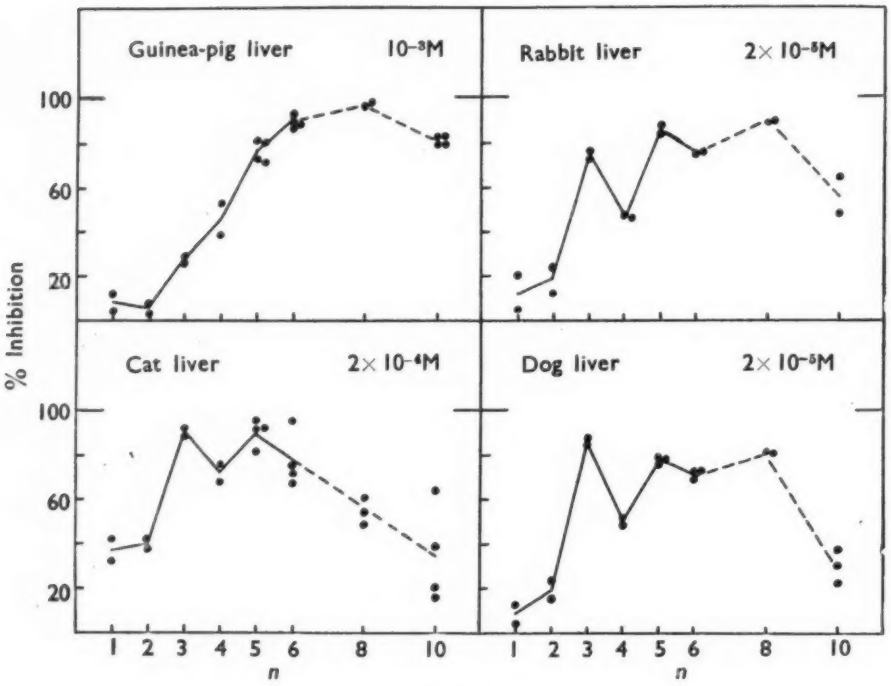

Guinea-pig liver
10⁻⁵M
Rabbit liver
2× 10⁻⁵M
Cat liver
2× 10⁻⁴M
Dog liver
2× 10⁻⁵M
% Inhibition
100
60
20
1 2 3 4 5 6 8 10
n

FIG. 1a

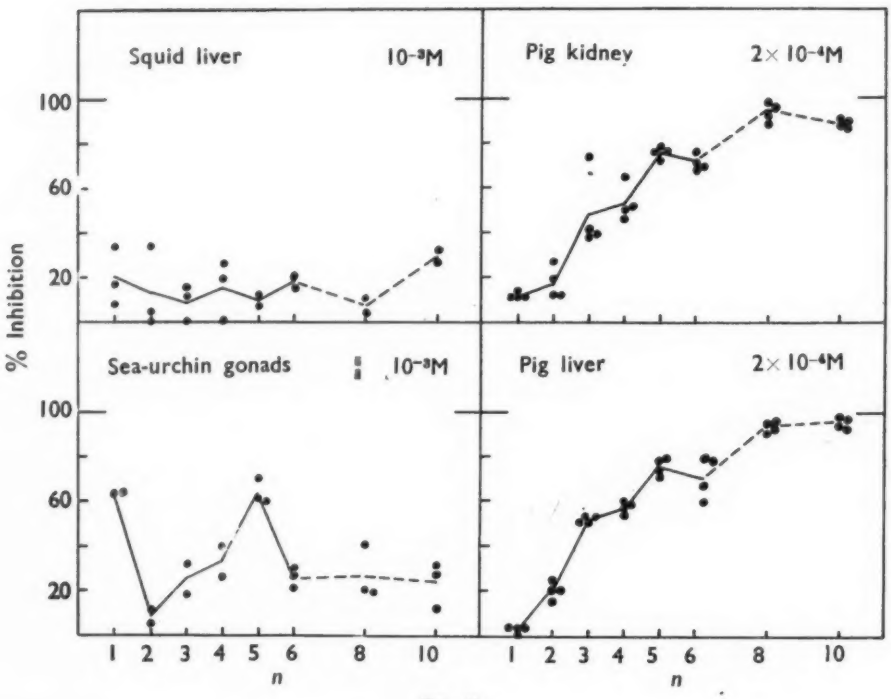

Squid liver
10⁻³M
Pig kidney
2× 10⁻⁴M
Sea-urchin gonads
10⁻³M
Pig liver
2× 10⁻⁴M
% Inhibition
100
60
20
1 2 3 4 5 6 8 10
n

FIG. 1b

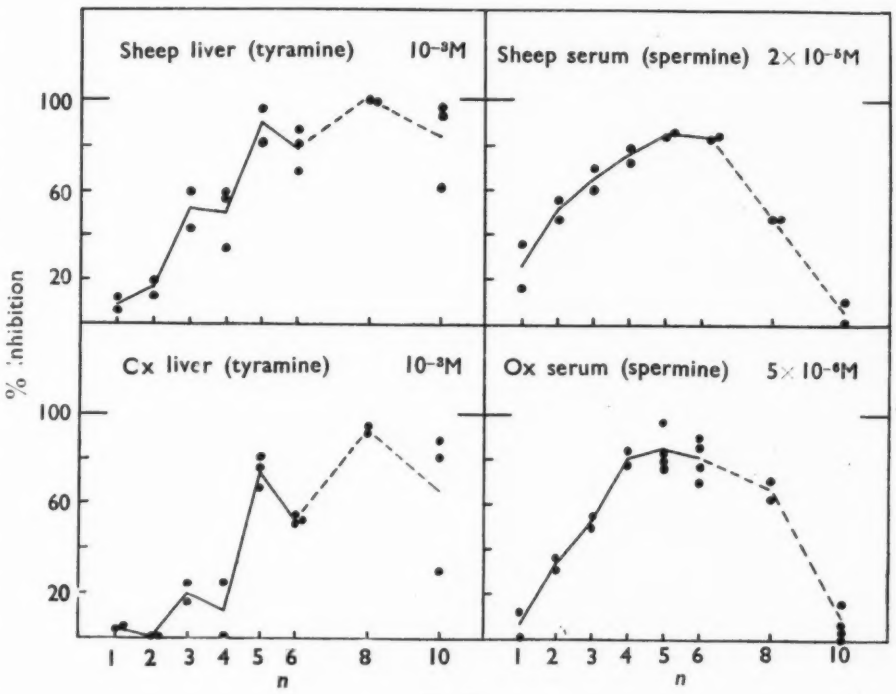


FIG. 1c

FIG. 1.—Inhibition of amine oxidase and of spermine oxidase by diamidines of the homologous series (I). Ordinates, percentage inhibition; abscissae, number (*n*) of carbon atoms in the polymethylene chain. Each diagram gives the source of enzyme and the molar concentration of inhibitor used. In all diagrams in Fig. 1a and 1b the substrate was tyramine; in those of Fig. 1c the substrate used is indicated in parentheses.

and of the dog (Fig. 1a) is very sensitive, whereas that of the guinea-pig (Fig. 1a) and of the squid (Fig. 1b) is rather insensitive.

(*b*) The relation between chain length and inhibitory activity also differs from species to species. The experiments with rabbit liver are in good agreement with the findings of Blaschko and Duthie (1945); the optima for $n=3$ and $n=5$ are again evident, and there appears to be a third optimum in the neighbourhood of $n=8$. The curve for dog liver is rather similar to that for rabbit liver (Fig. 1a). With pig (Fig. 1b) and guinea-pig (Fig. 1a) liver the long-chain members are more active than those with short chains. Enzyme from sea-urchin gonads has one optimum for $n=1$ and one for $n=5$ (Fig. 1b).

(*c*) Little difference was noted between the inhibitions of pig liver enzyme and pig kidney enzyme. Not only was there no significant difference between the percentage inhibitions but also the curves relating inhibition to chain length were very similar (Fig. 1b).

(*d*) Spermine oxidase, from ox and from sheep serum, was strongly inhibited by amidines (Fig. 1c); the optimum chain length with both preparations was in the region of $n=4$ to $n=6$, in marked contrast to the results with amine oxidase from these two species (Fig. 1c).

DISCUSSION

These results show that the amine oxidases from different species are different entities with differing inhibitor specificities. The results with pig tissues suggest that, at least in this species, enzyme from two different organs is not distinct. On the other hand, the two different enzymes from ruminants—amine oxidase and spermine oxidase—display entirely different inhibitor specificities within the same species.

SUMMARY

1. The inhibition of amine oxidase and of spermine oxidase from different species by members of a homologous series of diamidines has been studied.

2. Enzyme preparations from different species display marked differences in their sensitivity to amidines; moreover, the relation between the length of the polymethylene chain and inhibitory activity differs from species to species.

3. Spermine oxidase is strongly inhibited by some of the diamidines studied.

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THE ACTION OF ANTRYCIDE UPON TRYPANOSOMES *IN VITRO*

BY

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(RECEIVED JULY 19, 1955)

This paper reports experiments on the action of antrycide upon normal and resistant trypanosomes *in vitro*.

METHODS

The stock strain of *Trypanosoma equiperdum*, which has been maintained at this Institute for about thirty years, was used. An antrycide-resistant strain was prepared from it by treating infected mice with one or several sub-effective doses of antrycide and then passing the trypanosomes into fresh mice, and so on. After about six months, a high degree of resistance *in vivo* was obtained; a dose of 0.4 mg. antrycide/20 g. produced practically no effect upon the development of the infection, whereas with the normal parent strain 0.05 mg./20 g. was sufficient to clear the blood of trypanosomes.

The *in vitro* experiments were carried out by the technique of Yorke and Murgatroyd (1930) with some modifications. The medium consisted of equal parts of rabbit or horse serum and of Tyrode solution adjusted to approximately *p*H 7.2. This was distributed in 0.5 ml. amounts into small flat-bottomed tubes, closed with rubber bungs. Appropriate concentrations of antrycide methylsulphate were added to the Tyrode solution, three tubes being set up for each concentration. Approximately 0.05 ml. of washed trypanosome suspension was added to each tube, and the tubes were then incubated at 35° C. In order to test the infectivity of the trypanosomes for mice, the contents of a tube were injected intraperitoneally into one or two mice. The quantities of antrycide carried over with the inoculum were too small to have any significant effect.

RESULTS

Action Upon Normal Trypanosomes

During the course of many experiments trypanosomes (*T. equiperdum*) were consistently killed in 20 hr. by concentrations of antrycide ranging from 10^{-6} to 10^{-7}. Trypanosomes were not killed by these concentrations in 12 hr. When the period of observation was extended to 48 hr., the apparent trypanocidal power of the drug was not greatly increased. When trypanosomes were exposed to low concentrations of antrycide for a long enough period and then injected intraperitoneally into mice (approximately 100,000/mouse) the mice did not become infected although the trypanosomes were actively motile at the time of injection and although trypanosomes from control tubes without drug were normally infective. (This loss of infectivity is a common phenomenon when trypanosomes are exposed *in vitro* or *in vivo* to antrycide, suramin, or phenanthridinium compounds.) In the present experiments this loss of infectivity appeared to be a gradual process depending upon the length of exposure and the concentration of antrycide. Thus, in one experiment when the trypanosomes were exposed for 5 hr. at 35° C., the minimum concentration of antrycide which destroyed infectivity was 4×10^{-6} to 10^{-6}; but when they were exposed for 24 hr. the minimum effective concentration was 10^{-9}. In other experiments infectivity was destroyed when the trypanosomes were exposed for 1 hr. before injection although not if they were exposed for only half an hour. The gradual nature of the combination between drug and trypanosome was also demonstrated by experiments in which the trypanosomes were exposed to antrycide for a suitable period and then washed and set up again in nutrient medium at 35–37° C.; when the trypanosomes were removed from the antrycide (10^{-6}) in 6 or in 12 hr., they survived *in vitro* for a further 20 hr.

Action Upon Antrycide-resistant Trypanosomes

The action of antrycide upon normal and resistant strains *in vitro* is shown in Table I. The sensitivity of the two strains to the direct trypanocidal action of the drug was almost the same. Thus, the minimum trypanocidal concentration at 24 hr. was 10^{-6} to 2.5×10^{-7} for the normal strain and 4×10^{-6} to 10^{-6} for the antrycide-resistant strain, giving a ratio between the two strains of about 4. On the other hand, there was a marked contrast in the retention of infectivity by the two strains. After 5 hr. exposure the infectivity of

TABLE I
THE EFFECT OF ANTRYCIDE UPON TRYPANOSOMES *IN VITRO* AT 35° C.

Strain	Concn. of Antrycide	Trypanosomes					
		Start	5 Hr.		24 Hr.		48 Hr.
		No./mm.³	No./mm.³	Infectivity	No./mm.³	Infectivity	No./mm.³
Normal	1: 250,000	56	84	0/2	0		
	1:1 million		59	1/2 (11)	0		
	1:4 ,,		60	2/2 (8, 8)	3	0/1	0
	1:16 ,,		60	2/2 (3, 3)	44	0/1	22
	1:64 ,,		60	2/2 (2, 3)	62	0/1	33
	1:256 ,,	50	60	2/2 (2, 2)	56	0/2	25
	1:1024 ,,	72	45	2/2 (2, 2)	31	0/2	22
	Control	73	86	2/2 (2, 2)	53	1/1 (7)	16
Antrycide-resistant	1:250,000	27	19	2/2 (3, 4)	0		
	1:1 million	27	19	2/2 (2, 3)	1	0/1	
	1:4 ,,	27	31	2/2 (2, 2)	16	1/1 (9)	Contaminated
	1:16 ,,	27	20	0/2 (2, 2)	17	0/2	5
	1:64 ,,	27	20	2/2 (2, 2)	25	1/1 (6)	0
	1:256 ,,	27	20	2/2 (2, 2)	5	0/1	Contaminated
	Control	27	32	2/2 (2, 2)	23	—	14

The figures for " Infectivity " indicate the proportion of inoculated mice which became infected, and also (in parentheses) the number of days before trypanosomes appeared in their blood.

the normal strain was mostly destroyed at 4×10^{-6} and 10^{-6} and much diminished at 2.5×10^{-7} whereas the infectivity of the resistant strain was not appreciably affected by 4×10^{-6}. After 24 hr. the infectivity of the normal strain was destroyed by 10^{-9}, but some of the resistant trypanosomes were still infective after exposure to 2.5×10^{-7}.

DISCUSSION

According to the experiments described above, antrycide is effective in killing our strain of *T. equiperdum in vitro* at 35° C. at a concentration of 10^{-6} in 20 hr., and under our conditions it destroys their infectivity for mice in much lower concentrations. These findings differ considerably from those reported by Ormerod (1951) working in this laboratory with the same strain of trypanosomes. He found that the minimum trypanocidal concentration after 20 hr. was 5×10^{-5} to 1.25×10^{-5}.

The difference in the results is difficult to explain. In our experiments antrycide-resistant trypanosomes were almost as sensitive as the normal trypanosomes to the *in vitro* trypanocidal action of antrycide—this agrees with the similar finding by Ormerod, although at a different concentration. On the other hand, resistant trypanosomes were much less sensitive than normal ones to the effect of antrycide upon infectivity. In view of this behaviour of antrycide-resistant trypanosomes it is considered that the direct trypanocidal action of antrycide *in vitro* is probably not the explanation of its therapeutic effect *in vivo*, even though it takes place at concentrations comparable to those which are achieved *in vivo* (Spinks, 1950). On the other hand, the action of antrycide in causing loss of infectivity is probably important for its therapeutic action. Similar loss of infectivity is caused by suramin and phenanthridinium compounds, which resemble each other in their therapeutic effects. Perhaps these compounds suppress the multiplication of the trypanosomes.

SUMMARY

1. When trypanosomes (*T. equiperdum*) were exposed to antrycide methylsulphate *in vitro* at 35° C. for 20 hr., the minimum trypanocidal concentration of the drug was 10^{-6} to 2.5×10^{-7} for a normal strain, and only four times greater than this for an antrycide-resistant strain, which resisted the maximum tolerated dose *in vivo*.

2. The power of normal trypanosomes to infect mice was destroyed by *in vitro* exposure to antrycide 10^{-6} for 5 hr. at 35° C., but that of antrycide-resistant trypanosomes was hardly affected by 4×10^{-6}.

3. Judging by these results, the power of antrycide *in vitro* to destroy infectivity is probably more significant for its therapeutic action *in vivo* than is its direct trypanocidal action *in vitro*.

Grateful acknowledgments are due to Mr. K. Broomfield and to Miss M. Cameron for technical assistance.

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THE PARALYSING ACTION OF MORPHINE ON THE GUINEA-PIG ILEUM

BY

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(RECEIVED JULY 23, 1955)

Morphine has a paralysing action on the peristaltic reflex in isolated guinea-pig ileum, as first demonstrated by Trendelenburg (1917). Recent work (Schaumann, Giovannini and Jochum, 1952; Schaumann, 1954) has shown that all strong analgesics have this property and that their potency on this preparation runs parallel to their analgesic power. The *laevo* isomers are much more active than the *dextro* isomers, both as analgesics and on the guinea-pig gut. Levorphan, which is the strongest analgesic, is also the most effective on the peristaltic reflex; its *dextro* isomer is inactive in both tests. These findings suggest that the analgesics exert their specific paralysing action by a mechanism analogous to that which is responsible for their analgesic effects. If so, the guinea-pig ileum would be a suitable preparation on which to study the mode of action of morphine and its substitutes.

The present investigations were undertaken to determine the site of action of morphine on the guinea-pig ileum. Its paralysing activity was compared qualitatively and quantitatively with that of hexamethonium and atropine on the intestine stimulated by intraluminal pressure, nicotine, phenoxycholine, and acetylcholine. A few experiments were also done on the jejunum of the rabbit.

METHODS

Guinea-pigs or rabbits were killed by a blow on the neck. In the experiments on the guinea-pig the lower ileum was used, discarding the 10 cm. next to the caecum. A strip of jejunum was taken in the experiments on rabbits. The preparations were suspended in Mg-free Tyrode solution to which 0.3N-HCl was added to adjust the *p*H to 7.1–7.3. The bath was aerated with 95% O_2 and 5% CO_2 and the temperature kept at 36–37° C.

To elicit the peristaltic reflex the preparations were set up in a 45 ml. bath according to the method described by Trendelenburg (1917). Changes in volume, and isotonic contractions of the longitudinal muscle, were recorded on a smoked drum. Peristaltic waves were elicited by a rise in intraluminal pressure of 1.5 or 2 cm. Tyrode solution. In some experiments the gut was stored in Tyrode solution at 5.5° C. for 24 hr., then suspended at 37° C. and allowed to recover for at least 1 hr. before the experiment was started. For distension of the lumen a pressure of 3 or 4 cm. of Tyrode solution was used in these tests. In normal and in cooled preparations the rise in intraluminal pressure was maintained for 1 min. and followed by a rest period of 2 min.

Nicotine and phenoxycholine were added to the 45 ml. bath in doses of 30 or 45 μg., washed out after 45 sec. and given every 3 min.

Hexamethonium, atropine, and morphine were added 1 min. before a rise in intraluminal pressure or an application of nicotine or phenoxycholine; they were kept in the bath during several tests until their effect was maximal. The doses were increased from ineffective values in geometric steps of 1.5–2.0. The lowest dose was determined which inhibited peristaltic waves during a 1 min. rise in intraluminal pressure or reduced the contractions of the longitudinal muscle to less than 30%.

The drugs were used as the following salts: hexamethonium iodide, morphine sulphate, atropine sulphate, nicotine hydrogen tartrate, and β-dimethylamino-ethoxybenzene-iodomethylate (phenoxycholine iodate). All doses stated in the text refer to μg. of the salts added to the 45 ml. bath.

RESULTS

Guinea-pig Ileum

The peristaltic reflex is easily obtained in the guinea-pig intestine on distension of the lumen. It consists of two phases, termed by Trendelenburg preparatory and emptying. The preparatory phase consists of a contraction of the longitudinal muscle; the emptying phase, which follows, is a wave of contraction of the circular muscle, spreading aborally over the whole preparation. According to Feldberg and Lin (1949), different mechanisms are responsible for the two phases.

Emptying Phase.—It is known that the emptying phase of the peristaltic reflex is abolished by

*British Council Scholar.

ganglionic blocking drugs such as hexamethonium (Paton and Zaimis, 1949), by analgesics such as morphine, and by atropine (Trendelenburg, 1917). Comparing the weight-for-weight potencies of these drugs in abolishing the emptying phase, atropine was the most and hexamethonium the least active. Atropine abolished the emptying phase in doses of 0.1–2.0 μg., morphine in doses of 0.5–5.0 μg., and hexamethonium in doses of 30–250 μg. The mean values from the results, together with the standard error of the mean and the number of experiments, are given in Table I, which shows that atropine was about three times as active as morphine and about 120 times as active as hexamethonium. The effects of morphine and of hexamethonium are illustrated in Fig. 1.

Nicotine and Phenoxycholine.—The nicotine contraction was also abolished by atropine, morphine or hexamethonium. Again atropine was the most and hexamethonium the least active drug, but the relative potencies were not the same as for the abolition of the emptying phase; both atropine and morphine were active in smaller, and hexamethonium in larger, doses than on the emptying phase. Further, whereas atropine was about three times as active as morphine in abolishing the emptying phase, it was about five times as active in abolishing the nicotine contraction. The relative potencies are given in Table I.

The ganglion-stimulating action of phenoxycholine has been described by Lévy, Michel-Ber, and Cafiot (1953). Weight for weight phenoxycholine produced in the guinea-pig ileum a stronger contraction than nicotine. This contraction was also abolished by atropine, morphine and hexamethonium, in the same order of potency as with nicotine (Table I).

TABLE I

DOSES (μG.) OF ATROPINE SULPHATE, MORPHINE SULPHATE, AND HEXAMETHONIUM IODIDE WHICH BLOCK THE EMPTYING AND PREPARATORY PHASES OF THE PERISTALTIC REFLEX AND NICOTINE AND PHENOXYCHOLINE CONTRACTIONS OF THE GUINEA-PIG ILEUM PREPARATION SUSPENDED IN A 45 ML. BATH

(No. of experiments in parentheses)

	Atropine	Morphine	Hexamethonium
Emptying phase 1·5 or 2 cm.	0·89 ± 0·26 (8)	2·72 ± 0·61 (16)	104 ± 21 (11)
Nicotine 30 or 45 μg.	0·32 ± 0·05 (9)	1·50 ± 0·41 (10)	200 ± 39 (7)
Phenoxycholine 30 or 45 μg.	0·32 ± 0·13 (10)	2·70 ± 0·61 (13)	170 ± 60 (7)
Preparatory phase 3 or 4 cm.	0·5 ± 0·14 (5)	3·14 ± 0·38 (7)	No block

In some experiments the effect of atropine, morphine or hexamethonium was compared with equally strong contractions produced by nicotine or phenoxycholine. This is illustrated in the experiment of Fig. 2 in which 45 μg. phenoxycholine and 75 μg. nicotine were used. A given dose of morphine (3 μg.), of atropine (0.12 μg.), or of hexamethonium (not shown in the Fig.) reduced both contractions to the same extent. There was thus no quantitative difference in the paralysing action of these drugs on equal contractions produced by nicotine and phenoxycholine.

The effect of morphine in abolishing a nicotine contraction was overcome by increasing the

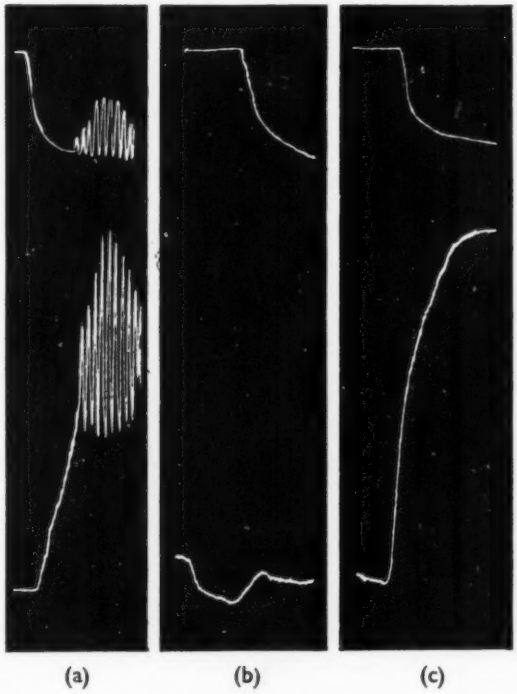

FIG. 1.—Guinea-pig ileum preparation suspended in 45 ml. Tyrode solution. In this and the following Figs. the upper tracing records intestinal volume, the lower tracing contractions of the longitudinal muscle. Intraluminal pressure raised to 2 cm. Tyrode soln. at (*a*), (*b*), and (*c*). At (*b*) in the presence of 3 μg. morphine and at (*c*) in the presence of 100 μg. hexamethonium.

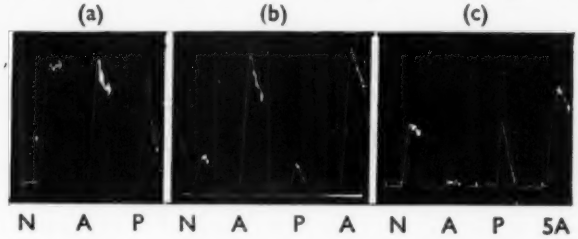

FIG. 2.—Guinea-pig ileum preparation suspended in 45 ml. Tyrode solution. Effect of morphine and atropine on equal contractions produced by 75 μg. nicotine (N), 0.18 μg. acetylcholine (A), and 45 μg. phenoxycholine (P). At (*b*), in the presence of morphine, at first 3 μg. (thin line) and later 15 μg. (thick line); at (*c*) in the presence of 0.12 μg. atropine; at (5A) dose of ACh increased to 0.9 μg.

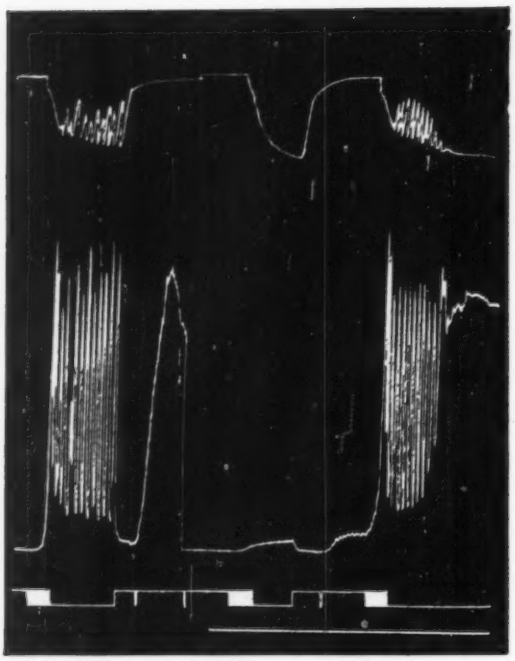

FIG. 3.—Guinea-pig ileum preparation suspended in 45 ml. Tyrode solution. Effect of morphine on peristalsis and nicotine contractions. The white marks at (*a*), (*d*), and (*f*) indicate that the intraluminal pressure was raised slowly to 2 cm. and maintained during the downward deflection of the signal. At (*b*) and (*e*) effects of 30 μg. nicotine; at (*c*) change of bath fluid. The bottom line indicates the presence of 3 μg. morphine in the bath.

nicotine concentration in the bath or by raising the intraluminal pressure of the intestine. This is illustrated in Fig. 3. It shows that 3 μg. morphine abolished the peristaltic reflex and the contraction produced by 30 μg. nicotine, but that when the pressure was raised in the presence of nicotine full peristaltic waves were again elicited. Similarly, peristaltic contractions blocked by morphine reappeared when nicotine was added whilst the pressure in the lumen was raised.

The Preparatory Phase.—Both atropine and morphine abolished the preparatory phase which, however, remained unaffected by hexamethonium. This difference between the action of morphine and hexamethonium on the peristaltic reflex is illustrated in Fig. 1, which shows that morphine abolished both the preparatory and the emptying phases, whereas hexamethonium abolished the emptying phase only. Previous storage of the preparation in the cold for 24 hr. had a similar effect to that of hexamethonium: it abolished the emptying phase but left the preparatory phase intact. On these preparations there was the same difference in the actions of hexamethonium and morphine. Hexamethonium in doses of up to 750 μg. was without any effect on the preparatory phase. On the other hand, morphine acted on these preparations in doses of 2–5 μg. Fig. 4 illustrates this effect of morphine and shows additional features. The tone of the muscle decreased on addition of small doses of morphine to the bath and any spontaneous movements ceased. When the intraluminal pressure was raised, the longitudinal muscle no longer contracted and the decrease in tone was then made further evident by a greater filling of the lumen, as seen from the volume record in Fig. 4. When the morphine was washed out, the tone not only reappeared but increased, particularly on raising the intraluminal pressure; the contraction of the longitudinal muscle was enhanced and the filling decreased. Further, when the intraluminal pressure was again lowered, the longitudinal muscle did not completely relax at once as in the untreated preparation; there was an initial, quick, partial relaxation followed by a prolonged period of gradual, slow relaxation so that the tone returned to normal only after about 5 min.

When the morphine was kept in the bath for a long time, its effect in abolishing the preparatory phase did not usually persist. This is illustrated in the experiment of Fig. 5, which shows the return of the preparatory phase in the continued presence of morphine in the bath. As seen from the volume record, the return of the preparatory phase was associated with a return of the normal distensibility of the intestine. When the preparatory phase had returned in the presence of small doses of morphine, addition of higher doses could again reduce it. This effect was transient and finally

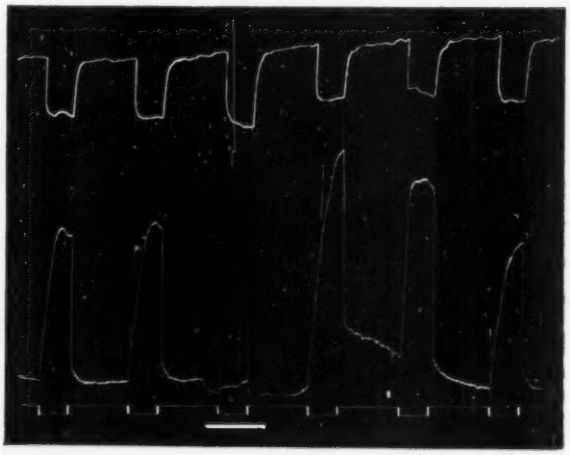

FIG. 4.—Guinea-pig ileum preparation, previously cooled for 24 hr., suspended in 45 ml. Tyrode solution. At signals, intraluminal pressure raised to 3 cm. for 1 min. The white line indicates the presence of 3 μg. morphine in the bath.

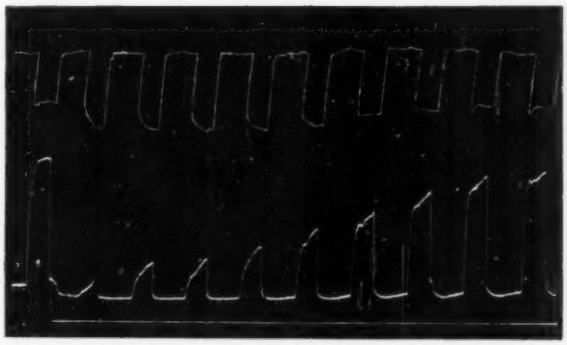

FIG. 5.—Guinea-pig ileum preparation, previously cooled for 24 hr., suspended in 45 ml. Tyrode solution. Effect of continued presence of 2 μg. morphine (white line) on the preparatory phase. Intraluminal pressure raised to 3 cm. for 1 min. every 3 min.

the preparatory phase became insensitive to morphine. Washing out the morphine in this condition produced no enhancement of the preparatory phase and no delay in the relaxation of the muscle.

The return of the preparatory phase in the continued presence of morphine in the bath did not occur in all experiments. In some there was only partial recovery, in others none at all.

Atropine, like morphine, abolished the preparatory phase in the cooled preparation. Weight for weight atropine was about six times as active as morphine; its action was more delayed, reached maximum within about 5 min., and then wore off in spite of its continued presence in the bath, and, as with morphine, the full preparatory phase was again obtained on raising the intraluminal pressure, which eventually could not be reduced by a tenfold increase in the dose of atropine. When atropine was washed out at a time when it was exerting its maximal effect, the sensitivity did not return at once to normal as after morphine; atropine never produced an enhancement of the preparatory phase or an increase in tone.

Acetylcholine.—The contractions produced by 0.1–0.3 μg. ACh were more sensitive to atropine than corresponding contractions produced by 30–75 μg. nicotine or by 30–45 μg. phenoxycholine. On the other hand, morphine did not abolish the ACh contractions (Fig. 2). In some experiments they were slightly reduced, but this reduction could not be increased by increasing the dose of morphine. It may be attributed to the slight ganglionic action of ACh described by Ambache (1946) and Feldberg (1951).

Rabbit Jejunum

Contractions elicited by *nicotine* are known to be relatively resistant to atropine (Lévy *et al.*, 1953). As shown in Fig. 6, 40 μg. atropine was required to prevent a response to 30 μg. nicotine, which was greatly reduced by 1 mg. hexamethonium. Morphine had no effect even when tested in doses of up to 1 mg.

The preparatory phase was studied in fresh preparations only. It was slightly reduced by 40 μg. atropine and was slightly increased by 400 μg. morphine (Fig. 6).

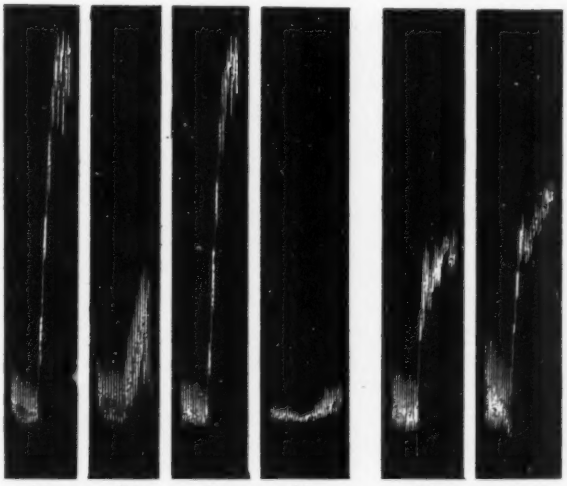

FIG. 6.—Rabbit jejunum preparation suspended in 45 ml. Tyrode solution. (*a*)–(*d*) Contractions produced by 30 μg. nicotine in the presence of 1 mg. hexamethonium at (*b*), of 1 mg. morphine at (*c*), and of 40 μg. atropine at (*d*). At (*e*) and (*f*) intraluminal pressure raised to 3 cm. in the absence (*e*), and presence (*f*), of 400 μg. morphine.

DISCUSSION

The present experiments show that the emptying phase of the peristaltic reflex and the contractions elicited by ganglion-stimulating drugs are abolished by about the same doses of atropine, morphine, or hexamethonium. Hexamethonium is a ganglion-blocking agent, and the results with hexamethonium thus agree with the assumption that stimulation of ganglion cells is involved in the emptying phase of the peristaltic reflex as well as in the nicotine contractions. The fact that atropine and morphine also abolish the emptying phase and the nicotine contractions, however, cannot be taken as evidence that they act as ganglion-blocking agents. Transmission in the superior cervical ganglion is readily blocked by hexamethonium, but atropine blocks only in high doses, and morphine, even in high doses, blocks only occasionally (Hebb and Konzett, 1950). Nevertheless, morphine might have a blocking action on the ganglion cells of the intestinal wall and not on the superior cervical ganglion, but there is no

necessity to postulate such an action to explain the effect of morphine on the emptying phase and on the nicotine contraction. Morphine may act by interrupting the pathway between ganglion cells and smooth muscle at a more peripheral point. This mode of action of morphine could explain why the preparatory phase—which is not blocked by hexamethonium and therefore not mediated by ganglion cells—is readily blocked by small doses of morphine. Further, as already shown by Henderson (1928), the preparatory phase is not affected by cooling, which abolishes the emptying phase of the peristaltic reflex. As Ambache (1946) found, the first effect of cooling an intestinal preparation is damage to the ganglion cells. The same argument as put forward for the site of action of morphine may apply for that of atropine, although it has some ganglion-blocking action in high concentrations, at least on the superior cervical ganglion.

We do not know the mechanism by which the preparatory phase is produced. Feldberg and Lin (1949) found that it was not abolished by hexamethonium or by cocaine, and therefore concluded that it is a myogenic response to a stretch of the longitudinal muscle fibres. This conclusion has not been generally accepted. There is, in fact, good evidence that stretching the longitudinal muscle does not in itself provoke a contraction, but that the preparatory phase is a reflex response to the stimulus of radial distension. Schaumann, Jochum, and Schmidt (1953) showed that when the longitudinal muscle was slowly stretched there was no increase in tone, and Kosterlitz, Pirie, and Robinson (1955), using isometric recording, proved that the increase in longitudinal tension produced by stretching the longitudinal muscle was insignificant compared with that elicited by distension of the lumen. A quick stretch also did not cause a contraction of the longitudinal muscle. Further, Schaumann, Jochum, and Schaumann (1953) found that the paralysing effect of morphine and its substitutes on the preparatory phase runs parallel with their analgesic and not with their spasmolytic activities ; indeed, not all of them have a spasmolytic action on the intestinal preparation (Haas, Hohagen, and Kollmannsperger, 1953).

It is therefore concluded that atropine and morphine act on the nervous elements involved in the preparatory phase. Nicotine and phenoxycholine probably stimulate some other nervous elements in addition to ganglion cells, and the resultant contraction may be mediated by the same nerve endings as the preparatory phase. These endings are probably the site of action of atropine and morphine. If we accept this concept, the finding by Feldberg and Lin (1949) that cocaine abolishes the emptying phase but not the preparatory phase of the peristaltic reflex suggests that cocaine in the doses used by these authors acted on the ganglion cells.

The effect of atropine and morphine in abolishing the preparatory phase of the peristaltic reflex need not necessarily be the result of the same mode of action of these drugs. Atropine could paralyse the preparatory phase by preventing the action of released acetylcholine ; if so, morphine, which is not an antagonist to acetylcholine, could act by preventing its release. Or both atropine and morphine could act on the same point in the efferent part of the reflex without the mediation of acetylcholine. This possibility may be less likely because of the difference of action of morphine and atropine on rabbit jejunum. On this preparation, only atropine has a paralysing effect on the preparatory phase and on nicotine contractions. Only guinea-pig intestine seems to be susceptible to the specific paralysing effect of morphine and its substitutes.

In addition to its action on the preparatory phase, morphine decreases the tone and abolishes spontaneous movements of the intestinal preparation. This may mean that these motor phenomena are also maintained by the same nervous elements as the preparatory phase. It is not possible at present to offer a satisfactory explanation for the two other effects of morphine in the present experiments on the intestinal preparation—tachyphylaxis, which also applies to atropine and which has also been observed *in vivo* for morphine and its substitutes (Schaumann, 1954), and the rise in tone which occurs after washing out the morphine.

Summary

1. Morphine and atropine abolish the preparatory and emptying phases of the peristaltic reflex and contractions produced by ganglion-stimulating drugs in isolated guinea-pig ileum.

2. The preparatory phase of the peristaltic reflex is still obtained when the ganglion cells have been inactivated. Therefore the paralysing effect of morphine cannot be attributed to paralysis of the ganglion cells in the intestinal wall.

3. It is suggested that atropine paralyses the preparatory phase by preventing the action of released acetylcholine, morphine by preventing its release ; or that atropine and morphine act on the same point in the reflex involved in the preparatory phase without the mediation of acetylcholine.

4. On the rabbit's jejunum morphine, in contrast to atropine, does not reduce the nicotine contraction or the preparatory phase of the peristaltic reflex.

I wish to express my gratitude to Dr. W. Feldberg for his invaluable help and advice, and to the Medical Research Council for their hospitality.

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THE REACTIVATION BY OXIMES AND HYDROXAMIC ACIDS OF CHOLINESTERASE INHIBITED BY ORGANO-PHOSPHORUS COMPOUNDS

BY

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Many organo-P-compounds, such as dialkylphosphorofluoridates, tetraalkylpyrophosphates and dialkyl *p*-nitrophenylphosphates, are potent inhibitors of cholinesterase (ChE). In contrast to other specific inhibitors such as eserine and neostigmine, their action is non-competitive and not readily reversible. It is now generally believed that these inhibitors phosphorylate some vital group at the active centre of the enzyme (Aldridge, 1954 ; Wilson, 1954) and that reversal of inhibition requires the rupture of the phosphorus-enzyme bond.

Inhibition by compounds containing a dimethoxyphosphoryl group is reversed moderately quickly by water (Aldridge and Davison, 1953), whereas inhibition by compounds containing a diethoxyphosphoryl group is reversed only slowly by water (Aldridge and Davison, 1953) but more readily by other nucleophilic reagents such as choline and, more particularly, hydroxylamine (Wilson, 1952). It was later shown that hydroxamic acids were considerably more effective than hydroxylamine in reactivating TEPP-inhibited ChE and would also reactivate ChE inhibited by di*iso*propylphosphorofluoridate (dyflos), which is resistant to reactivation by either water or hydroxylamine (Wilson and Meislich, 1953 ; Wilson and Ginsburg, 1955).

These results suggested that other compounds containing an ionizable NO—H group might be effective reactivating agents. Simple oximes are only weakly acidic, but many *iso*nitroso compounds are known which are much stronger acids and which have now been shown to be very effective reactivating agents for ChE inhibited by TEPP, dyflos and *iso*propyl methylphosphonofluoridate (Sarin). A number of hydroxamic acids have also been studied for comparison, but they are generally less potent than the *iso*nitroso compounds.

METHODS

Red-cell Experiments.—Washed human red cells were suspended in an equal volume of saline and allowed to stand in contact with a dilute solution of the inhibitor (for concentration see Tables) for 10 min. at 25° C. The cells were then twice washed with ice-cold saline to remove excess of the inhibitor and stored at 0° C. until immediately before use. One volume of the cell suspension was then mixed with one volume of the reactivating agent in "barbitone buffer"* (as used later in the enzyme assay procedure) and stored at 25° C. At intervals, 1.0 ml. of this mixture was added to 10.0 ml. acetylcholine (0.01M) in barbitone buffer. ChE determinations were by the electrometric method (Michel, 1949).

Rat Brain Experiments.—Rats were killed by cervical dislocation. The brain was removed immediately and the adhering membranes were dissected off. Each g. of tissue was dispersed in 4 ml. saline (0.85%) by means of a Potter-Elvehjem homogenizer. The homogenate was used within 1 hr. of preparation. The dilute aqueous inhibitor (0.05 ml.—for concentration see Tables) was added to the enzyme dispersion (2.0 ml.) and the mixture was allowed to stand for 2 hr. at 25° C. One volume of homogenate was then mixed with one volume of reactivating agent in barbitone buffer, and after either 1 or 24 hr. an aliquot was withdrawn and assayed for ChE activity by the electrometric method.

With both types of experiment controls were run upon the enzyme alone, upon the enzyme in the presence of the reactivator, and upon the inhibited enzyme.

The percentage reactivation was calculated from the following expression :

$$\frac{\text{Activity of Reactivated Enzyme} - \text{Activity of Inhibited Enzyme}}{\text{Activity of Enzyme Control} - \text{Activity of Inhibited Enzyme}} \times 100$$

In general, at 0.01M, the reactivating agent did not depress the activity of the enzyme control by more than about 10%, so that the maximum possible percentage reactivation was over ninety. If the reactivation be calculated from the enzyme activity in the presence of the reactivator, the numbers given in the Tables would generally be a little higher.

* Barbitone Na, 0.01M; KH_2PO_4, 0.002M; KCl, 0.3M.

Materials.—The oximes and hydroxamic acids used in this investigation were synthesized by two of us (A. F. C. and A. L. G.). They were characterized by melting-point determinations and nitrogen analyses.

RESULTS

Reactivation of TEPP-inhibited ChE

Table I shows the effect of a number of *iso*-nitroso compounds on human red cell ChE inhibited by TEPP. Picolinhydroxamic acid and nicotinhydroxamic acid methiodide—the most effective hydroxamic acids reported by Wilson and Ginsburg (1955)—are included for comparison together with pyrimidine-2-hydroxamic acid, a new compound, which appears to be more active than either of these.

TABLE I

THE REACTIVATION OF ChE INHIBITED BY TEPP

The ChE of washed human red cells was inhibited by contact with 10^{-6}M-TEPP for 10 min. at 25° C. Excess TEPP was removed with ice-cold saline. The inhibited cells were incubated at 25° C. with solutions of reactivating agent in barbitone buffer at *p*H *circa* 7.4. Aliquots were withdrawn at intervals and the ChE activity determined by the Michel electrometric method.

Agent	Concn. (M)	% Reactivation in		
		¼ hr.	1 hr.	6 hr.
Di*iso*nitrosoacetone (DINA) ..	·01	58	78	—
Mono*iso*nitrosoacetone (MINA) ..	·01	53	97	—
*iso*Nitrosoacetophenone (INAP) ..	·01	49	83	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	11	24	84
Glyoxime	·01	1	8	23
Diacetylmonoxime	·01	—	11	40
Pyrimidine-2-hydroxamic acid ..	·01	50	92	100
Picolinhydroxamic acid	·01	37	83	100
Nicotinhydroxamic acid methiodide	·05	10	33	76

Reactivation of Dyflos-inhibited ChE

A wide range of oximes and hydroxamic acids has been tested against ChE inhibited by dyflos. Table II gives the results of screening tests on red blood cells.

TABLE II

THE REACTIVATION OF ChE INHIBITED BY DYFLOS

Method as in caption to Table I. Inhibitor, 10^{-6}M-dyflos.

Agent	Concn. (M)	% Reactivation in		
		1 hr.	3 hr.	6 hr.
Phenyl glyoxime	·01	50	64	67
*iso*Nitrosoacetophenone (INAP) ..	·01	45	61	66
Di*iso*nitrosoacetone (DINA) ..	·01	32	58	64
Mono*iso*nitrosoacetone (MINA) ..	·01	15	40	56
p-Methoxy*iso*nitrosoacetophenone	·005	7	15	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	4	4	8
Glyoxime	·01	1	2	3
Diacetylmonoxime	·01	0	0	0
Acetoxime	·01	0	0	0
Picolinhydroxamic acid	·05	44	56	—
Nicotinhydroxamic acid	·01	2	6	11
Nicotinhydroxamic acid methiodide	·01	3	8	15
Salicylhydroxamic acid	·01	2	11	17

A few other compounds were tested in a preliminary way against dyflos-inhibited rat brain ChE. Some of these at concentrations between 0.01M and 0.1M showed about 50% reactivation after 24 hr.: these were salicylaldoxime, benzhydroxamic acid and furohydroxamic acid. The following compounds showed no activity even after contact for 24 hr.: α- and β-furfuraldoximes, *p*-hydroxybenzaldoxime, *p*-aminobenzhydroxamic acid and hippurohydroxamic acid.

Di*iso*nitrosoacetone (DINA) showed similar activity towards both rat brain and red cell cholinesterase inhibited by dyflos.

Reactivation of Sarin-inhibited ChE

Table III shows results for several oximes and hydroxamic acids against sarin-inhibited red blood cell ChE. The *iso*nitroso compounds are generally more effective against ChE inhibited by sarin than against ChE inhibited by TEPP. This is in marked contrast to water and hydroxylamine, which will reactivate ChE inhibited by TEPP but not by sarin, and picolinhydroxamic acid, which is far more effective against ChE inhibited by TEPP than against ChE inhibited by sarin.

TABLE III

THE REACTIVATION OF ChE INHIBITED BY SARIN

Method as in caption to Table I. Inhibitor, 10^{-7}M-sarin.

Agent	Concn. (M)	% Reactivation in		
		¼ hr.	1 hr.	6 hr.
Di*iso*nitrosoacetone (DINA) ..	·01	94	100	—
Mono*iso*nitrosoacetone (MINA) ..	·01	89	100	—
*iso*Nitrosoacetophenone (INAP) ..	·01	30	65	—
*iso*Nitrosoacetylacetone (INAA) ..	·01	8	33	69
Glyoxime	·01	2	7	23
Nicotinhydroxamic acid methiodide	·05	11	39	54
Picolinhydroxamic acid	·05	5	20	55

Several compounds have been tested against both red blood cell and rat brain ChE inhibited by sarin. As with dyflos the results were similar irrespective of enzyme preparation.

A wide range of compounds were initially examined for ability to reactivate rat brain ChE inhibited with sarin. All the compounds in the four groups below were tested at 0.01M, at 25° C. and at a *p*H of approximately 7.4.

Group 1. Over 50% reactivation in 1 hr.: DINA, *iso*nitrosoacetophenone (INAP), mono*iso*nitrosoacetone (MINA), phenylglyoxime, pyridine-2-aldoxime methiodide, and pyridine-4-aldoxime methiodide.

Group 2. About 50% reactivation in 24 hr.: (*a*) *Oximes.* Di*iso*nitroso*cyclo*hexanone, glyoxime, *iso*-nitrosoacetylacetone (INAA), *iso*nitrosoacetylfuran, isatin-β-oxime, pyridine-2-aldoxime, pyridine-4-aldoxime, salicylaldoxime, and tri*iso*nitrosopropane.

(*b*) *Hydroxamic acids.* Nicotinhydroxamic methiodide, picolinhydroxamic acid, and pyrimidine-2-hydroxamic acid.

Group 3. About 20% reactivation in 24 hr.: (*a*) *Oximes.* *o*-Chlorobenzaldoxime, diacetylmonoxime, methylglyoxime, pyridine-3-aldoxime methiodide, and pyrogallaldoxime. (*b*) *Hydroxamic acids.* Furohydroxamic acid and salicylhydroxamic acid.

Group 4. Less than 10% reactivation in 24 hr.: (*a*) *Oximes.* Acetoxime, cyclohexane-1:2-dionedioxime, gallacetophenone oxime, glucose oxime, *o*-hydroxyacetophenone oxime, *m*- and *p*-hydroxybenzaldoximes, *iso*nitrosodimedone, pentane-2:3:4-trione-2:3-dioxime, pyridine-3-aldoxime, and pyrrole-2-aldoxime. (*b*) *Hydroxamic acids.* Benzhydroxamic acid, hippurohydroxamic acid, *iso*nicotinhydroxamic acid, *p*-methylbenzhydroxamic acid, and tropohydroxamic acid.

Discussion

Early work on the mechanism of ChE inhibition by phosphorus anticholinesterases suggested that the process of inhibition was irreversible. That this was not entirely true followed from the fact that ChE inhibited by TEPP did slowly recover some of its activity spontaneously, and later work by Aldridge and Davison (1953) on the spontaneous recovery of ChE after inhibition by diethoxy- and, more particularly, dimethoxyphosphoryl compounds showed that the reversal was a first order chemical reaction with an activation energy corresponding to that of the direct hydrolysis of the P-compounds themselves. These results agreed with the theory of ChE action developed by Wilson (1954) in which the inhibition was considered to occur by direct phosphorylation of some group at the active centre of the enzyme. Reversal occurred by displacement of the enzymic group from the phosphorus atom by another nucleophilic compound. A wide range of nucleophilic reagents was found by Wilson (1951) to be moderately effective in reversing inhibition by TEPP, but ineffective in reversing inhibition by dyflos. The later finding that hydroxamic acids could reverse inhibition by dyflos as well as by TEPP (Wilson and Meislich, 1953; Wilson and Ginsburg, 1955) supported the phosphorylation theory of the inhibitory process. The compounds described in the present paper are generally more effective than the hydroxamic acids in reactivating ChE inhibited by TEPP or dyflos, and markedly more effective in reactivating ChE inhibited by sarin—which, as with dyflos, is not reactivated by either water or hydroxylamine.

There is considerable variation in activity in compounds of a given chemical type against different inhibitors. The *iso*nitroso compounds are most effective against sarin, slightly less effective against TEPP, and least effective against dyflos. On the other hand, whereas picolinhydroxamic acid is nearly as effective as the oximes against TEPP and dyflos, it is only weakly effective against sarin, whilst nicotinhydroxamic acid methiodide shows similar activity to picolinhydroxamic acid against sarin, but is much less potent against either TEPP or dyflos.

When the same group of compounds is considered against different inhibitors the order of effectiveness does not remain the same. Thus from the results in Tables II and III it may be seen that the order of activity with dyflos is INAP$>$DINA$>$MINA whereas with sarin it is DINA $\simeq$ MINA$>$INAP. It is difficult, at present, to see any reason for this. Similarly with the hydroxamic acids, listed for their reactivation of sarin-inhibited rat brain ChE, there is considerable difference between the activity of those in sub-groups 2, 3 and 4, yet chemically and structurally there is apparently no more in common between the members of individual groups than between those of different groups. In the wide range of compounds tested, the only common factor among the most effective members is a pK_a value of around 8. It is probable that the reactivation, being a nucleophilic reaction, occurs through the anion of the oxime, so that, since the tests are carried out at *p*H 7.4, if the pK_a of the oxime is much higher than 7.4 the fraction of oxime ionized will be too small for reactivation to occur at a significant rate, whereas if the pK_a is much lower than 7.4—as with *iso*nitrosodimedone which has a *p*K of 5.7—although all the material will be ionized, the anion will be too weakly nucleophilic to be an efficient reactivator. From the screening results on sarin-inhibited rat brain ChE, and from the variation of activity among the same group of compounds with different inhibitors, it is obvious that, although this factor is concerned, it is not the decisive one. This is particularly so where the hydroxamic acids are concerned. Steric factors are, no doubt, also important and may account for the significantly lower activity of INAA compared with MINA and INAP, but it is difficult to see a reason why picolin- and pyrimidine-2-hydroxamic acids are more active than nicotin-, *iso*nicotin-, and benzhydroxamic acids, since sterically the compounds are similar—except for the presence of the α-nitrogen atom in the two active compounds—and the dissociation constants of all five compounds are of the same order.

SUMMARY

1. Compounds of a new series—the oximes—have been shown to be potent reactivators of both red cell and rat brain ChE inhibited by TEPP, sarin or dyflos.

2. These compounds are generally superior to the hydroxamic acids, particularly against sarin-inhibited ChE.

3. Factors influencing the relative activity of different compounds against different inhibitors have been discussed.

We wish to thank Miss June Petts for assistance with the biochemical work and Messrs. G. L. Sainsbury and C. Stratford for help with the preparation of many of the compounds examined.

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STIMULANT ACTIVITIES OF QUATERNARY AMMONIUM COMPOUNDS ON MAMMALIAN SKELETAL MUSCLE

BY

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Though much information is available about the nicotine-like stimulant activities of quaternary ammonium compounds on vasomotor ganglia, little quantitative work has been done with such compounds on mammalian skeletal muscle.

Simonart (1932) compared the stimulant activities of some choline esters and ethers on the denervated gastrocnemius muscle of the cat. He used a cannula in the inferior mesenteric artery as a means of introducing the drug close to the muscle. Of the compounds examined he found the esters (acetyl, propionyl, and *n*-butyryl) to be much more active than the ethers (methyl, ethyl, vinyl, and butyl).

On injecting acetylcholine (ACh) into the innervated gastrocnemius muscle, through a cannula in the tibial artery, Brown, Dale and Feldberg (1936) obtained regular contractions. Their technique was also used by Bacq and Brown (1937) to compare the activities of some quaternary ammonium compounds. Brown *et al.* (1936) and Bacq and Brown (1937) concluded that ACh is very active as a stimulant of skeletal muscle, in contrast to its low stimulant activity on the blood pressure.

The present work was undertaken to extend and confirm these observations—especially for various new nicotine-like stimulant compounds prepared in this laboratory by Hey (1952, 1954) and previously investigated by him from the point of view of the blood pressure, on which many are extremely active.

As distinct differences have been noted between "red" and "white" muscles in their responses to stimulant drugs (Riesser, 1921 ; Zaimis, 1951) and to neuromuscular blocking agents (Paton and Zaimis, 1949, 1951 ; Zaimis, 1951), a method was devised to record simultaneously the contractions caused by the close-arterial injection of the stimulant compounds to both "white" (gastrocnemius) and "red" (soleus) muscle.

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METHODS

The following compounds were investigated : propionylcholine, *n*-butyrylcholine, *n*-valerylcholine, *iso*-butyrylcholine, trimethylacetylcholine, 4-keto-amyltrimethylammonium, *n*-amyltrimethylammonium, choline ethyl ether, choline butyl ether, choline phenyl ether, choline *p*-chlorophenyl ether, choline *m*-bromophenyl ether, and nicotine.

Cats anaesthetized with chloralose (100 mg./kg.) and treated with intraperitoneal atropine sulphate (2 mg./kg.) were used throughout.

Activity of Nicotine-like Stimulant Compounds on Skeletal Muscle

The drugs were administered by a method similar to that of Brown, Dale, and Feldberg (1936). Muscle contractions were recorded with a Brown-Schuster mammalian muscle myograph, provision having been made for the simultaneous recording of the contractions of both gastrocnemius and soleus muscles. The sciatic nerve was stimulated by periodic condenser discharges (4/min.) through shielded platinum electrodes. During chemical stimulation of the muscle one electrical impulse was omitted. Stimulant drugs were injected retrogradely into the cannulated stump of the cut anterior tibial artery ; during the injection the popliteal artery was occluded. A constant injection volume of 1 ml. was used throughout. All drugs were made up in saline with a *p*H of about 5. The doses were measured in micromoles. Before each injection the cannula in the tibial artery was filled, using a fine hypodermic needle, with a solution of the drug in the same concentration as that about to be administered.

The response of the muscle was estimated as the ratio of the increased tension produced by the injection of the stimulant drug to that produced by the electrical stimulus. The initial tension in all experiments was maintained at 50 g.

Some of the compounds produced a paralysis of the muscles. The activities of the compounds which caused little or no paralysis were estimated by a (2 + 2) assay technique. Acetylcholine was used as the standard drug throughout the assays ; two doses of ACh were compared with two doses of the drug under test. These four doses were injected in random order ; this series of four injections was repeated, again in random order.

The activities of the compounds which caused a long-lasting or permanent paralysis were compared with ACh by a (2 + 1) assay technique. Two doses of ACh were compared with one dose of the test drug, which, on the basis of previous observations, would be expected to produce a response lying between the two responses to ACh.

Inhibition of True and Pseudo-Cholinesterases

Two specific anticholinesterase drugs were used to inhibit cholinesterase activity—iso-ompa (Austin and Berry, 1953a, b) and 284C51 (Austin and Berry, 1953a, b; Fulton and Mogey, 1954). Using the enzymes of human blood, Austin and Berry found that iso-ompa irreversibly inactivated the plasma pseudo-cholinesterase. Fulton and Mogey showed that 284C51 produced a reversible inhibition of true cholinesterase and had little or no effect on pseudo-cholinesterase.

Iso-ompa (25 mg./kg.) was injected via the femoral vein at the beginning of the experiment. 284C51 (0.25 mg./kg.) was also injected by this route at the beginning of each assay—that is, about every 30 min. —since Fulton and Mogey showed that intraperitoneal doses of this order produced an anticholinesterase action in mice which lasted at least half an hour.

Estimation of Cholinesterase Levels. — Cholinesterase activity was determined manometrically at 37.5° C. using open manometers and Warburg manometer flasks, with a bicarbonate-Ringer solution as the medium, equilibrated with 5% carbon dioxide and 95% nitrogen. Gastrocnemius muscle, homogenized in bicarbonate-Ringer so that 5 ml. of final suspension was equivalent to 1 g. of the original muscle, was used as the source of true cholinesterase, and oxalated plasma as the source of pseudo-cholinesterase. The substrates were acetylcholine, acetyl-β-methylcholine, and butyrylcholine, the final concentrations being 0.02M, 0.03M, and 0.02M respectively. The following quantities were placed in each flask: substrate 0.3 ml.; muscle homogenate 2.0 ml.; oxalated plasma 0.2 ml. with ACh and butyrylcholine as substrates, or 1.5 ml. with acetyl-β-methylcholine as substrate. The total volume was always adjusted to 3.0 ml. with bicarbonate-Ringer.

For the determination of the initial cholinesterase levels in plasma and in muscle, after the administration of atropine and chloralose, the tissues were dealt with in the following way: A sample of blood from the carotid artery was oxalated, centrifuged, and the plasma removed. The right gastrocnemius muscle was removed from the animal, washed with saline, lightly dried with filter paper, and weighed. Both plasma and muscle were stored overnight in a refrigerator at −5° C. The same procedure was adopted with blood and with the left gastrocnemius muscle after the injection of the two anticholinesterase drugs. The estimation of the initial and final cholinesterase levels was made on the following day.

Results

Nature of the Relation between the Dose of Acetylcholine (ACh) and the Response of Skeletal Muscle

There has been little quantitative work on the response of skeletal muscle to nicotine-like stimulant drugs; the first point investigated, therefore, was the nature of the relation between the dose and response of skeletal muscle to repeated injections of ACh.

Four different doses of ACh were given via the anterior tibial artery, in 4 series of randomized blocks, and the resultant contractions of the soleus and gastrocnemius muscles recorded. Data from this experiment are tabulated in Tables I and II. Statistical analyses, shown in Tables Ia and IIa, indicate that the regression of response on log. dose is highly significant ($P<0.001$), and

TABLE I

RELATIONSHIP BETWEEN DOSE OF ACh AND RESPONSE OF THE SOLEUS MUSCLE

Dose of ACh (μg.)	10	20	30	40	
Log dose = x ..	1·0	1·301	1·477	1·602	$\bar{x}$ = 1·345
	Response = y				Totals
Series 1	0·562	0·781	0·976	1·073	3·392
,, 2	0·415	0·658	0·775	0·850	2·698
,, 3	0·333	0·425	0·606	0·768	2·132
,, 4	0·200	0·389	0·526	0·677	1·792
Totals	1·510	2·253	2·883	3·368	$\bar{y}$ = 0·626

Regression coefficient b = 0·765

TABLE Ia

ANALYSIS OF THE DATA IN TABLE I

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Regression ..	0·47930	1	0·47930	352·43	< 0·001
Linearity ..	0·00599	2	0·00299	2·20	0·2
Between doses ..	0·48529	3	0·16176	118·94	< 0·001
,, expts. ..	0·36787	3	0·12262	90·16	< 0·001
Residual error ..	0·01225	9	0·00136		
Total	0·86541	15			

TABLE II

RELATIONSHIP BETWEEN DOSE OF ACh AND RESPONSE OF GASTROCNEMIUS MUSCLE

Dose of ACh (μg.)	10	20	30	40	
Log dose = x ..	1·0	1·301	1·477	1·602	$\bar{x}$ = 1·345
	Response = y				Totals
Series 1	0·438	0·688	1·188	1·375	3·689
,, 2	0·286	0·760	1·000	1·136	3·182
,, 3	0·569	0·569	0·863	1·118	3·119
,, 4	0·200	0·600	0·706	1·020	2·526
Totals	1·493	2·617	3·757	4·649	$\bar{y}$ = 0·782

Regression coefficient b = 1·297

TABLE IIa
ANALYSIS OF THE DATA IN TABLE II

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Regression ..	1·37528	1	1·37528	97·82	<0·001
Linearity ..	0·03557	2	0·01778	1·26	>0·2
Between doses ..	1·41085	3	0·47028	33·45	<0·001
,, expts. ..	0·17003	3	0·05667	4·03	0·05
Residual error ..	0·12658	9	0·01406		
Total	1·70746	15			

is also linear (P>0.2) for both the soleus and gastrocnemius muscles, taking P=0.05 as the level of significance in these and in all subsequent analyses. Fig. 1 shows the dose-response relation obtained for the soleus muscle; this illustrates a significant (P<0.001) reduction in the sensitivity to the chemical stimulus as the experiment proceeds.

It is of interest that the slopes of the regressions for ACh on the gastrocnemius and soleus muscles are significantly different (P<0.001); in this experiment the dose-response graph for ACh on the gastrocnemius muscle is steeper than that on the soleus muscle. This, however, does not always hold. In some subsequent experiments this state of affairs was reversed, and in others there was no significant difference between the regressions.

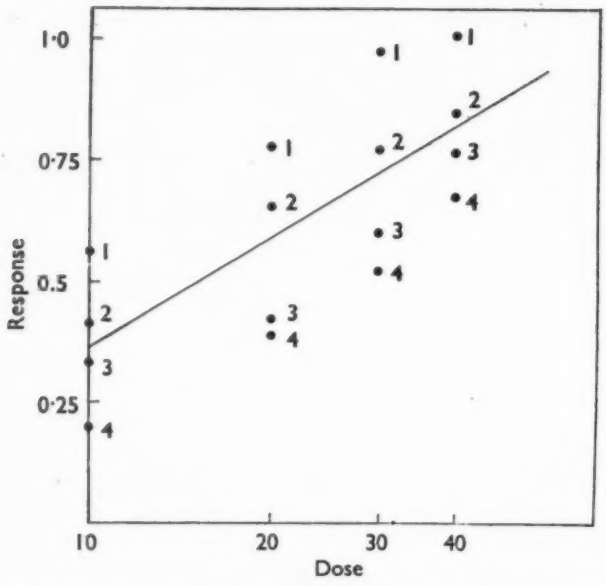

FIG. 1.—Dose-response curve to acetylcholine bromide on soleus muscle of a cat under chloralose anaesthesia. The numerals 1 to 4 refer to the successive series of doses. Injections were given into the anterior tibial artery. Electrical stimulation was by periodic condenser discharges (4/min.). Abscissa: dose of AChBr in μg. (log scale). Ordinate: response of muscle, estimated as the ratio of the increased tension produced by injection of the drug to that produced by the electrical stimulus. Note the decreased sensitivity to the chemical stimulus as the experiment proceeds.

Activity of Nicotine-like Stimulant Compounds on Skeletal Muscle

The (2+2) assay technique was used for the following compounds: propionylcholine, *n*-butyrylcholine, *iso*butyrylcholine, *n*-valerylcholine, trimethylacetylcholine and 4-keto-amyltrimethylammonium. Over the dose-range used both trimethylacetylcholine and 4-keto-amyltrimethylammonium produce a paralysis lasting approximately 30 min.; but, in contrast to those compounds estimated by the (2+1) assay technique, the muscles show complete recovery. Fig. 2 shows a typical record in which ACh was compared with *n*-valerylcholine. This record also demonstrates the transient paralysis produced by *n*-valerylcholine, a feature shown to a lesser extent by *iso*butyrylcholine, *n*-butyrylcholine and propionylcholine. Data from these assays were analysed, and the slope and parallelism of the regressions tested. Only in occasional assays was there any significant deviation from parallelism; such assays were discarded.

The (2+1) assay technique was used for the remaining compounds tested—that is, *n*-amyltrimethylammonium, choline ethyl ether, choline butyl ether, choline phenyl ether, choline *p*-chlorophenyl ether, choline *m*-bromophenyl ether, and nicotine. Fig. 3 shows a typical result, obtained with this technique, in which ACh is compared with choline phenyl ether. All these compounds cause a paralysis of approximately the same duration in the doses used, indicating a clear correlation between stimulating and paralysing activities.

From each assay the logarithm of the potency relative to ACh was determined. For those nicotine-like stimulant drugs whose activity was estimated by the (2+2) assay technique, the following formula (Burn, Finney, and Goodwin, 1950) was used to determine the logarithm of the relative potency:

$$M = \bar{x}_S - \bar{x}_T - \frac{\bar{y}_S - \bar{y}_T}{b}$$

where M is the logarithm of the relative potency, $\bar{x}_S$ and x_T are the mean values for the log dose of standard and test drug respectively, $\bar{y}_S$ and $\bar{y}_T$ are the mean responses, and b is the common regression coefficient. In the analysis of the (2+1) assays the assumption was made that the dose-response lines are parallel—an assumption not unfounded, since analysis of the data from the (2+2) assays shows that in most of the experiments there is no significant deviation from parallelism (P>0.05).

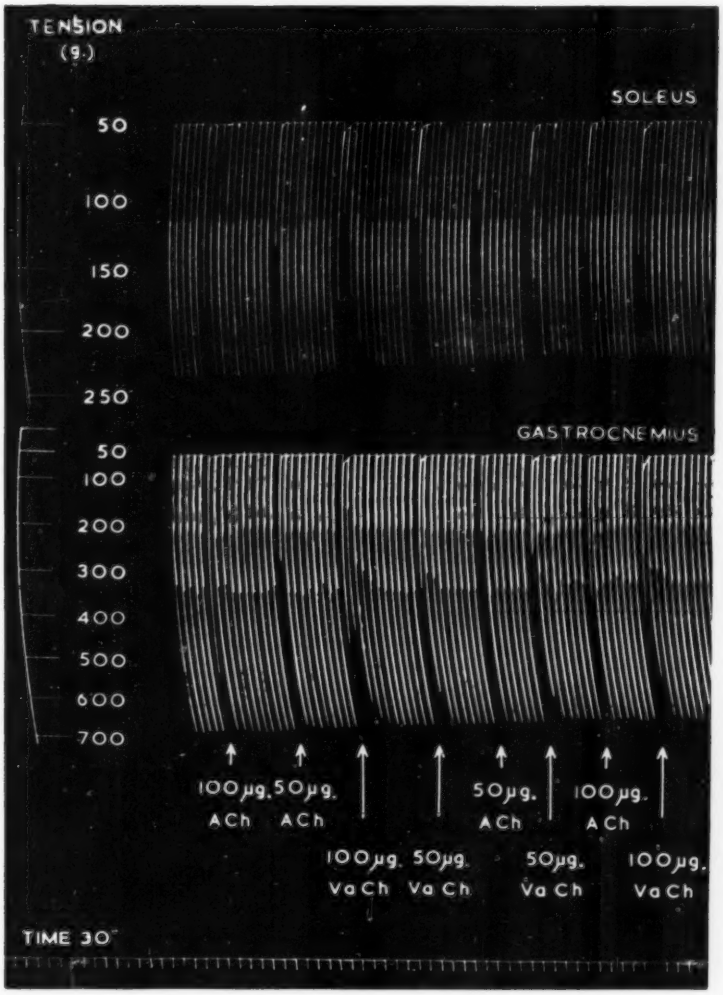

FIG. 2.—Comparison of acetylcholine and *n*-valerylcholine using the (2+2) assay technique. Cat, 3·2 kg., chloralose, atropine. Injections made into the anterior tibial artery during cessation of the supramaximal electrical stimuli applied to the sciatic nerve.

A complete list of the individual estimated potencies is given in Table III; Table IIIa shows the analysis of these results. Table IV shows the mean relative potencies and the limits of error (P=0.95) calculated from the residual mean square in Table IIIa. It should be noted that the last seven drugs in Table III were all estimated by a (2+1) assay, the test drug being injected after the two ACh injections, and as the response decreases as the experiment proceeds (Tables Ia and IIa), the estimates may all be too low; for this reason the limits of error have not been described as fiducial limits.

The analysis in Table IIIa reveals that there is no significant difference between the log relative potencies of these compounds when determined on either the soleus or the gastrocnemius muscle, and so the estimates of potency on the two muscles were grouped together.

Effect of Anticholinesterase Drugs on Relative Potencies

Before these results are discussed it must be remembered that the standard drug (ACh) undergoes enzymatic hydrolysis *in vivo*, whereas some of the test drugs do not. Hence the potency of the test drug may be altered if enzymatic hydrolysis is prevented. The experimental results recorded in this section show that prevention of the hydrolysis does not alter the relative potencies.

The drugs selected for this investigation were: propionylcholine, an ester rapidly hydrolysed by cholinesterases; trimethylacetylcholine, an ester causing paralysis of the muscles following preliminary stimulation; choline ethyl ether, a straight-chain ether; choline phenyl ether, and 4-keto-amyltrimethylammonium. The individual potencies were estimated graphically and are recorded in Table V.

A series of *t* tests was performed to determine whether there was any significant difference between the values of *M* for normal animals and for those treated with the two anticholinesterases. Taking P=0.05 as defining the level of significance, there is no significant difference between the mean relative potencies on the treated and untreated animals in four of the five tests of significance; the fifth test, on trimethylacetylcholine, borders on significance (P=0.04). Too much weight should not, however, be attached to this last test, as there is no reason to expect a significant difference between treated and untreated animals for trimethylacetylcholine and not for the other compounds. A joint probability was therefore derived from the results of the individual tests of significance, using the method described by Fisher (1944); this gives P=0.2–0.3.

There is thus no significant difference between the relative potencies estimated on the skeletal muscles of normal animals and on animals treated with anticholinesterase drugs.

Manometric Estimation of the Cholinesterase Levels in Skeletal Muscle and Oxalated Plasma.—In every experiment the dose of iso-ompa used

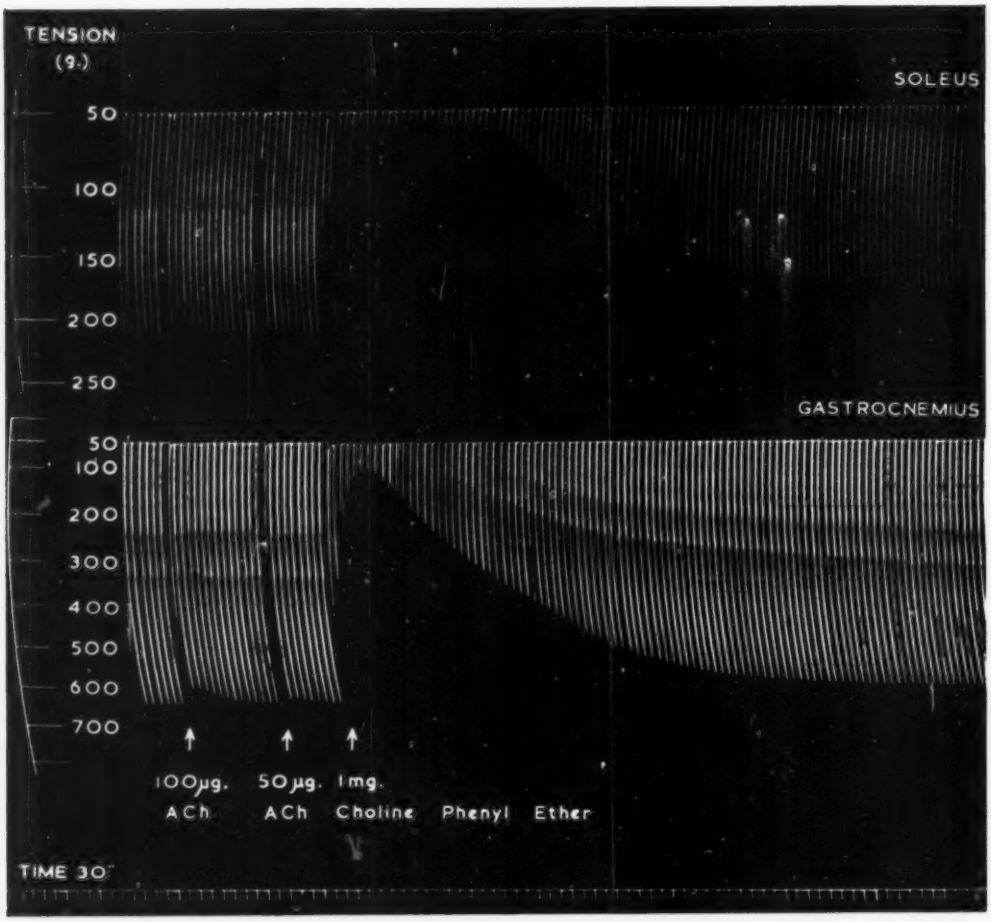

FIG. 3.—Comparison of acetylcholine and choline phenyl ether using the (2+1) assay technique. Cat, 3·2 kg., chloralose, atropine. Injections made into the anterior tibial artery during cessation of the supramaximal electrical stimuli applied to the sciatic nerve.

TABLE III

LOG POTENCIES RELATIVE TO ACh OF ALL THE NICOTINE-LIKE STIMULANT COMPOUNDS INVESTIGATED, DETERMINED ON THE SOLEUS AND GASTROCNEMIUS MUSCLES

Drug	Muscle	Log Individual Estimates of Potency					
Propionylcholine iodide	Soleus	+0·117	+0·131	+0·088	−0·008	−0·012	
	Gastrocnemius	+0·173	+0·136	+0·097	−0·039	−0·026	
n-Butyrylcholine ,,	Soleus	+0·097	−0·225	−0·078	+0·006		
	Gastrocnemius	+0·188	−0·008	−0·077	−0·016		
n-Valerylcholine ,,	Soleus	+0·048	+0·127	−0·043	−0·107		
	Gastrocnemius	−0·045	+0·189	−0·061	+0·070		
*iso*Butyrylcholine ,,	Soleus	−0·468	−0·357	−0·396			
	Gastrocnemius	−0·330	−0·399	−0·393			
Trimethylacetylcholine iodide	Soleus	−0·778	−0·918	−0·772			
	Gastrocnemius	−0·918	−0·797	−0·852			
4-Keto-amyltrimethylammonium iodide	Soleus	−0·979	−0·970	−0·775			
	Gastrocnemius	−0·655	−1·002	−0·727			
n-Amyltrimethylammonium iodide	Soleus	−0·796	−0·987	−0·758	−0·939	−0·704	
	Gastrocnemius	−0·604	−1·355	−0·758	−0·959	−0·815	
Choline ethyl ether bromide	Soleus	−1·448	−1·182	−1·117	−1·049	−1·340	
	Gastrocnemius	−1·157	−1·133	−1·230	−0·982	−1·322	
Choline butyl ,, ,,	Soleus	−1·893	−1·453	−1·658	−1·721	−1·544	
	Gastrocnemius	−1·890	−1·554	−1·442	−1·570	−1·431	
Choline phenyl ,, ,,	Soleus	−0·978	−1·263	−1·261	−1·103	−1·375	
	Gastrocnemius	−0·992	−1·301	−1·090	−0·954	−1·379	
Choline *p*-chlorophenyl ether bromide	Soleus	−2·251	−3·016	−3·120	−3·164	−3·104	−2·962
	Gastrocnemius	−2·222	−3·050	−3·012	−3·041	−3·161	−2·824
Choline *m*-bromophenyl ,, ,,	Soleus	−0·598	−0·669	−0·722	−0·936	−0·919	
	Gastrocnemius	−0·763	−0·574	−0·861	−0·706	−0·758	
Nicotine acid tartrate	Soleus	−1·255	−1·778				
	Gastrocnemius	−1·100	−1·372				

TABLE IIIa

ANALYSIS OF LOG RELATIVE POTENCIES OF TABLE III

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Between drugs ..	79·61094	12	6·63424	213·05	<0·001
,, muscles	0·05588	1	0·05588	1·79	0·1–0·2
Residual error ..	2·98986	96	0·03114		
Total	82·65668	109			

TABLE IV

POTENCIES RELATIVE TO ACh=1, AND APPROXIMATE LIMITS (P=0·95), OF THE NICOTINE-LIKE STIMULANT COMPOUNDS ON CAT SKELETAL MUSCLE

Drug	Mean Relative Potency (ACh=1)	Approximate Limits
Propionylcholine iodide	1·164	0·901 to 1·503
n-Butyrylcholine ,, ...	0·967	0·727 ,, 1·288
n-Valerylcholine ,, ...	1·052	0·791 ,, 1·401
*iso*Butyrylcholine ,, ...	0·407	0·293 ,, 0·566
Trimethylacetylcholine iodide ..	0·145	0·104 ,, 0·202
4-Keto-amyltrimethylammonium iodide	0·141	0·101 ,, 0·196
n-Amyltrimethylammonium iodide	0·136	0·105 ,, 0·175
Choline ethyl ether bromide ..	0·0637	0·0493 ,, 0·0822
,, butyl ,, ,, ..	0·0242	0·0188 ,, 0·0313
,, phenyl ether bromide ..	0·0677	0·0524 ,, 0·0874
,, *p*-chlorophenyl ether bromide	0·00123	0·000973 ,, 0·00155
Choline *m*-bromophenyl ether bromide	0·178	0·138 ,, 0·229
Nicotine acid tartrate	0·0420	0·0281 ,, 0·0630

TABLE V

RELATIVE POTENCIES, AND APPROXIMATE LIMITS (P=0·95), OF CERTAIN NICOTINE-LIKE STIMULANT COMPOUNDS ON SKELETAL MUSCLE OF CATS PRETREATED WITH ISO-OMPA AND 284C51

Drug	Mean Relative Potency (ACh=1)	Approximate Limits
4-Keto-amyltrimethylammonium iodide	0·137	0·107 to 0·177
Trimethylacetylcholine iodide ..	0·222	0·173 ,, 0·286
Propionylcholine iodide ..	0·890	0·692 ,, 1·145
Choline ethyl ether bromide ..	0·054	0·042 ,, 0·070
,, phenyl ,, ,, ..	0·070	0·054 ,, 0·090

TABLE VI

INHIBITION OF CAT SKELETAL MUSCLE AND OXALATED PLASMA CHOLINESTERASES BY 284C51 AND ISO-OMPA

μl. CO_2/30 min./0·4 g. muscle or 0·2 ml. plasma

Expt. No.	Enzyme Source	Acetylcholine		Acetyl-β-methylcholine		Butyrylcholine	
		Normal	Treated	Normal	Treated	Normal	Treated
1	Plasma	114·9	20·2	101·9	56·3	247·0	−5·6
	Muscle	51·9	43·9	44·7	34·7	7·6	0·8
2	Plasma	82·1	26·2	95·6	63·0	176·3	−13·0
	Muscle	71·5	53·5	46·0	31·0	19·5	3·4
3	Plasma	87·1	−0·6	66·8	−1·9	149·5	−4·2
	Muscle	61·1	29·9	30·8	14·1	26·2	−0·4

produced complete inhibition of the pseudocholinesterase of blood plasma, indicated by the non-hydrolysis of butyrylcholine (Table VI). In two of the three experiments the true cholinesterase of blood plasma was only partially inhibited, as indicated by the reduced rate of hydrolysis of acetyl-β-methylcholine. Inhibition of the true cholinesterase of muscle was also incomplete. However, the inhibition with 284C51 is reversible, and, as the concentration of substrate in the manometric determinations was many times greater than the *in vivo* concentration, inhibition *in vivo* is expected to be greater than in the manometric estimations.

DISCUSSION

It is interesting to compare the nicotine-like stimulant activities of these quaternary ammonium compounds on cat skeletal muscle with their activities on the blood pressure of the spinal cat. These comparisons are shown in Table VII. Hey's (1952, 1954) estimates on the blood pressure were obtained from cats under chloralose anaesthesia. I determined the pressor activities of the other compounds on spinal cats.

TABLE VII

POTENCIES, RELATING TO ACh=1, OF NICOTINE-LIKE STIMULANT COMPOUNDS ON CAT BLOOD PRESSURE AND SKELETAL MUSCLE

Drug	Activity on Blood Pressure	Activity on Skeletal Muscle
Acetylcholine bromide	1·00	1·000
Propionylcholine iodide	1·55*	1·164
n-Butyrylcholine ,, ...	2·10*	0·968
n-Valerylcholine ,, ...	2·25*	1·052
*iso*Butyrylcholine ,, ...	3·80*	0·407
Trimethylacetylcholine iodide	10·1*	0·145
Choline phenyl ether bromide	82·9	0·068
Choline *p*-chlorophenyl ether bromide	7·16	0·001
Choline *m*-bromophenyl ,, ,, ...	319	0·178
Choline butyl ether bromide	1·88	0·024
Choline ethyl ,, ,, ...	0·56	0·064
4-Keto-amyltrimethylammonium iodide ..	24·2	0·141
n-Amyltrimethylammonium bromide	8·55	0·136
Nicotine acid tartrate	7·96	0·042

* Activity estimations obtained by Hey (1952; 1954).

None of the straight-chain choline esters investigated differs significantly from ACh in its stimulant activity on skeletal muscle. However, the mean values for the activity on the blood pressure show a gradual increase as the chain length is increased.

Successive replacement of the hydrogen atoms in the acetyl group of ACh has a very marked effect. Whereas replacement of one of the hydrogen atoms—as in propionylcholine—does not produce a significant difference in activity on skeletal muscle, the replacement of a second hydrogen

atom—*iso*butyrylcholine—increases the activity on the blood pressure but diminishes that on skeletal muscle to a highly significant extent ($P<0.001$). Replacement of all the hydrogen atoms—trimethylacetylcholine—again increases the activity on the blood pressure while diminishing that on skeletal muscle ($P<0.001$).

Although substituted choline phenyl ethers are many times more active than ACh on the blood pressure and much less active on skeletal muscle, these activities are related. Choline *m*-bromophenyl ether is most active on the blood pressure and skeletal muscle; choline phenyl ether is next in order of activity, and choline *p*-chlorophenyl ether is least active on both effectors. Moreover, if the activity of choline *m*-bromophenyl ether on both the blood pressure and skeletal muscle is given an arbitrary value of 1, then the activity of choline phenyl ether relative to choline *m*-bromophenyl ether on the blood pressure becomes similar to its relative activity on skeletal muscle. An analogous relation exists between choline *m*-bromophenyl ether and choline *p*-chlorophenyl ether.

When the effect of chain structure on the activities of these compounds as stimulants of blood pressure and of skeletal muscle contraction is considered, little apparent relation exists between the activities on the two effector structures. Whereas 4-keto-amyltrimethylammonium and *n*-amyltrimethylammonium do not differ significantly in their activities on skeletal muscle, the stimulant activity of 4-keto-amyltrimethylammonium on the blood pressure is much greater than that of *n*-amyltrimethylammonium. Choline butyl ether and choline ethyl ether do, however, show a type of relation similar to that of the series of compounds representing the successive replacement of the hydrogen atoms in the acetyl group of ACh. Choline butyl ether is more active than choline ethyl ether on the blood pressure, but is significantly less active ($P<0.001$) on skeletal muscle.

These results are thus extremely complex. Some of the compounds within a series show certain relations between their stimulant activities on skeletal muscle and on blood pressure. Other series, such as those having the same chain length as ACh (4-keto-amyltrimethylammonium, *n*-amyltrimethylammonium and choline ethyl ether), show no apparent relation.

Even where there is a relation—as in the substituted choline phenyl ethers, and in the series of compounds representing the replacement of the hydrogen atoms in the acetyl group of ACh by methyl groups—this is not straightforward. The substituted choline phenyl ethers show a corresponding increase in stimulant activity on both the blood pressure and skeletal muscle, whereas the effect of hydrogen atom replacement in ACh is to increase the stimulant activity on the blood pressure but to diminish the activity on skeletal muscle.

It appears that the chain length of the molecule is of little importance to blood-pressure stimulating activity. One possible factor influencing this activity may be the electron density on the oxygen atom of choline compounds—as suggested by Hey (1952)—the activity increasing as the electron density is reduced.

Certain series of the compounds described do show increasing activity as the chain length is increased—for example, the straight-chain esters and ethers. This effect may possibly be caused by an increase in the lipoid solubility of the molecule, consequent on the increase in size of the alkyl groups, resulting in the "active spots" or "receptors" being made more easily accessible. The theory of Hey (1952) does not account for the difference in activity between choline butyl ether and choline ethyl ether, since the electron density on the oxygen atom is not expected to be much different in these two compounds; the lipoid solubility hypothesis would, however, account for this difference. Similarly, the lipoid solubility of the group attached to the ether oxygen atom can explain the increase in activity on the blood pressure and the decrease in activity on skeletal muscle shown by the two series consisting of propionylcholine, *iso*butyrylcholine and trimethylacetylcholine on the one hand, and choline ethyl ether and choline butyl ether on the other. The activity on the blood pressure increases in each series as the size of the alkyl groups is increased, whereas the activity on skeletal muscle—which contains little lipoid material in comparison with vasomotor ganglia—is diminished as the size of the alkyl groups is increased.

These concluding remarks are, however, largely speculative, and further work alone can determine their worth.

Summary

1. The nicotine-like stimulant activities of some quaternary ammonium compounds have been investigated on (*a*) the blood pressure and (*b*) the gastrocnemius ("white") and soleus ("red") muscles of the cat.

2. The potencies of these compounds, relative to acetylcholine, do not differ significantly when determined on either the soleus or the gastro-

cnemius muscle ; nor are these potencies affected by previous injection of anticholinesterase drugs.

3. The straight-chain choline esters—acetylcholine, propionylcholine, *n*-butyrylcholine and *n*-valerylcholine—do not differ significantly in their activities on skeletal muscle, but the pressor activity increases as the chain length is increased.

4. Successive replacement of the hydrogen atoms in the acetyl group of acetylcholine diminishes the activity on skeletal muscle while increasing that on the blood pressure.

5. Nuclear substitution in choline phenyl ether leads to changes in activity on skeletal muscle which parallel the changes in activity on the blood pressure. Choline *m*-bromophenyl ether has the highest activity, choline *p*-chlorophenyl ether the least, and that of choline phenyl ether is intermediate.

6. Compounds with the same chain length as acetylcholine—choline ethyl ether, 4-keto-amyltrimethylammonium and *n*-amyltrimethylammonium—show no apparent relation between their activities on blood pressure and on skeletal muscle.

7. The possible inferences from these results are discussed.

This work was done during the tenure of a Wellcome Pharmaceutical Research Fellowship. I wish to thank Professor W. A. Bain for his helpful encouragement and suggestions throughout this work ; my other colleagues in the department for many fruitful discussions and assistance ; Professor P. Hey for kindly providing me with the compounds investigated, and Sir Charles Lovatt Evans and Dr. A. C. White for the iso-ompa and 284C51 respectively.

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THE USE OF SOME MOLLUSCAN HEARTS FOR THE ESTIMATION OF 5-HYDROXYTRYPTAMINE

BY

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This work was undertaken mainly in order to find a sensitive method for the assay of 5-hydroxytryptamine (HT) in tissue extracts. The biological test mostly used has been the contraction of oestrous rat uterus (Erspamer, 1942 ; Amin, Crawford, and Gaddum, 1954), the sensitivity of which lies between 1 and 10 μg./l. This organ is sensitive to other substances, which may be present in tissue extracts, such as adrenaline which inhibits it and oxytocin and bradykinin which stimulate it. Paper chromatographic methods have been described, and that of Jepson and Stevens (1953) detects quantities nearly as small as does the rat uterus, but lack of specificity limits its use.

Erspamer and Ghiretti (1951) demonstrated that enteramine, later identified as HT, stimulated isolated molluscan heart, especially that of *Helix pomatia* and *Octopus vulgaris*. This was confirmed with synthetic HT on octopus heart (Bacq, Fischer, and Ghiretti, 1952). These preparations, however, were not found to be suitable for assay purposes. In the hands of Zetler and Schlosser (1954) *Helix pomatia* heart gave satisfactory results. Welsh (1953) found that *Venus mercenaria* (clam) heart—which had been earlier shown by him (Welsh, 1943) to be suitable for the quantitative estimation of acetylcholine (ACh) in tissue extracts—was stimulated by HT in low concentrations. This heart was used by Twarog and Page (1953) for the determination of HT in tissue extracts. The threshold concentration was 0.2–2 μg./l. and the heart was relatively insensitive to other substances except ACh, the effect of which can, however, be blocked by benzoquinonium (Mytolon) (Ludueña and Brown, 1952), which is used clinically to paralyse voluntary muscles.

Of preparations from different marine invertebrates found on the south coast of Britain, Welsh (1954) suggested the hearts of *Cyprina islandica* and *Buccinum undatum* as the most promising for HT and ACh bioassays. The heart of the freshwater mussel, *Anodonta cygnea*, also reacts to HT with graded responses (Fänge, 1955).

As mentioned above ACh is active on molluscan hearts and the usual response is inhibition. Recently Pilgrim (1954) has studied the action of ACh on the heart of a number of lamellibranch molluscs and Hughes (1955) has described *Mya arenaria* as a sensitive preparation for the assay of ACh. Adrenaline has been reported to stimulate some molluscan hearts in low concentrations, but has often been found quite ineffective. The results of different workers even on the same species have been different. For further information about the pharmacology of the molluscan heart the review of Krijgsman and Divaris (1955) may be consulted. These preparations are sometimes surprisingly different from the vertebrate heart or plain muscle.

This paper describes the actions of certain drugs on the isolated hearts of various molluscs living in the sea near Britain, on the hearts of two snails (*Helix aspersa* and *H. pomatia*), and on preparations of *Lumbricus terrestris*. The heart of the bivalve, *Spisula solida*—formerly known as *Mactra solida*—was found most useful, because of the relative abundance and hardiness of the animal, the constancy of its heart over long periods and its sensitivity to HT. It has the advantage for workers in Great Britain that it is much more easily available that *Venus mercenaria*.

METHODS

In most of the experiments the heart was suspended at room temperature (13–21° C.) in a 2 ml. bath, the contents of which could be changed by overflow, so that the mechanical disturbance was small. The heart of *Buccinum* was cannulated with a Straub cannula through the aorta and the auricle was tied off. Air was always bubbled through the fluid and the movements of the heart were recorded on a smoked drum with a light lever.

Bivalves were opened by cutting both adductor muscles with a thin scalpel. Usually the technique of dissection was essentially that of Welsh and Taub (1948); threads were tied near the auriculo-ventricular junctions and a small piece of ventricle was included in the ligature because of the fragility of the auricles. The modification of this method proposed by Hughes (1955) was occasionally used, but appeared to be less effective when the heart was sluggish.

The ventricles of *Helix aspersa* and *Helix pomatia* were tied by ligatures in the ventriculo-aortic and auriculo-ventricular grooves to the light lever and a fixed support; a small piece of the auricle was retained because this seemed to produce a more regular beat.

The crop and gizzard of *Lumbricus terrestris* were suspended together in the same way, and in other experiments strips (2 mm. broad) of longitudinal or circular muscle from the body wall in front of the clitellum were used. The longitudinal strips were taken from the dorsal or ventral midline and all visible nerve chains were removed.

In most of the experiments with marine animals the heart was suspended in a solution of the following composition (g./l.): NaCl 23; Na_2SO_4 4.0; KCl 0.65; $CaCl_2$ 1.1; $MgCl_26H_2O$ 10; $NaHCO_3$ 0.2. Some hearts, which were relaxed and irregular, gave a better beat when the concentration of Mg was reduced 2–5 times. Sometimes at least the sulphate ion seemed to be necessary for regular contractions. Glucose (0.5–1 g./l.) was sometimes also included, but it did not appear to make any difference and was generally excluded. For the heart of *Mya* the solution was that recommended by Welsh and Taub (1948); for *Lumbricus* it was that recommended by Wu (1939), and for *Helix* it was that recommended by Zetler and Schlosser (1954).

To start an inert heart or revive a weak and irregular one the ergot alkaloids, such as ergometrine particularly, have been used (Welsh, 1953; Hughes, 1955). This treatment does not change the sensitivity to acetylcholine, but possibly as a consequence of the increased tonus and the stronger and quicker beat the effects of stimulant drugs such as HT are often obscured.

During preliminary tests in the Gatty Marine Laboratory the molluscs were kept in running sea-water. In this department *Spisula* was kept in cool sea-water (ca. 1 l./animal, changed twice a week) from two weeks to two months. Frequent changes of the sea-water did not seem to increase the lifetime of these animals. Usually the whole supply, collected at one time and place, survived equally long under varying conditions; aeration of the sea-water was not of any obvious advantage. In the same circumstances *Cardium* survived for about the same time. When packed in fresh seaweed and kept in a refrigerator *Spisula*, *Cardium*, and *Mya* survived 1–2 weeks. *Helix* was kept alive in glass jars at room temperature for 5 months or longer.

Drugs were added to the fluid surrounding the heart in the bath and usually left there for 1 min. The interval was dependent on the dose, but the addition of HT, for instance, could usually be repeated as soon as the original beat was again reached. The cycle in HT assays was from 2 to 4 min. unless doses about 100 times larger than the threshold dose were used.

The following drugs were used: bradykinin (M. Rocha e Silva), substance P (Amin and Crawford), 5-benzyloxygramine and 6-methylgramine (Glaxo Laboratories), dihydroergotamine methane sulphonate, lysergic acid diethylamide (LSD), "Hydergine"—a mixture of 3 dihydro ergot alkaloids (Sandoz Products), benzoquinonium chloride (Mytolon), noradrenaline, isoprenaline (Sterling Winthrop Research Institute), histamine acid phosphate, physostigmine sulphate, atropine sulphate, tryptamine hydrochloride, carbachol (The British Drug Houses), hexamethonium bromide (May and Baker), adenosine-5-phosphate (L. Light and Co.), 5-hydroxytryptamine creatinine sulphate (Abbott Laboratories), decamethonium (Allen and Hanburys), acetylcholine chloride (Roche Products), ergometrine maleate, tubocurarine chloride, ouabain (Duncan, Flockhart and Co.), adrenaline, pituitary (posterior lobe) extract (Burroughs Wellcome and Co.), dibenamine (Smith, Kline, and French).

Doses of 5-hydroxytryptamine, tryptamine, adrenaline, noradrenaline, acetylcholine, and histamine are given in terms of the base. Other doses refer to the corresponding salts, although the anion is not mentioned in the text. Doses are in terms of the final concentration in the bath, generally as μg./l.

RESULTS

Experiments with Drug Solutions

The reaction, if any, of the various hearts to HT was always stimulation. Small doses increased the force of the beat and larger doses increased the rate as well. The effect on the force of the beat was generally used to compare different responses. Tryptamine had the same action as HT in concentrations 100 times as great or more (in experiments with *Spisula*, *Cardium*, *Cyprina*, and *Helix*). When the heart was sensitive to ACh the response to small doses was a decrease of the force and rate of the beat. Different salines did not have any clear effect on the response to HT.

Lumbricus terrestris (*Earthworm*)

The crop and gizzard preparation contracted in the presence of HT (10 μg./l.) or ACh (1 μg./l.). It was difficult to obtain graded responses especially with HT, and spontaneous activity had a disturbing effect. The body wall muscles were resistant to HT in concentrations up to 10,000 μg./l.

No satisfactorily beating heart could be obtained from *Barnea candida*, *Mactra corallina*, *Mytilus edulis* (mussel) or *Paphia saxatilis*.

Zirphaea crispata

Graded responses to HT were obtained with concentrations from 1 to 1,000 μg./l.

Solen siliqua (*Razor Shell*)

The threshold concentration of HT for the hearts of *Solen siliqua* was about 1 μg./l., and although they reacted with graded responses with concentrations up to 1,000 μg./l., the three hearts tried were not beating constantly. ACh was active in the same concentrations and ergometrine and LSD stimulated the heart.

Buccinum undatum (*Whelk*)

The cannulated *Buccinum undatum* heart reacted to HT in a concentration of 1 μg./l., but often a dose 100 times higher was ineffective and the responses decreased during the experiment. Welsh (1954) obtained satisfactory results with this preparation, but in our experiments the heart beat was generally irregular and the myocardium was liable to rupture after vigorous beats so that the fluid leaked out and the experiment was spoiled. These animals were difficult to keep alive for more than 1–2 days after transportation to our department.

Cyprina islandica

This shell fish is about 10 cm. long and has a relatively large heart, which beat in the bath with a rate of 10 or less/min. HT and ACh were active in concentrations of 1 μg./l. Noradrenaline and adrenaline stimulated the heart and the threshold concentrations were 1,000 and 5,000 μg./l. respectively. Histamine (5,000 μg./l.) was inactive.

LSD in concentrations from 0.1 to 100 mg./l. increased the amplitude and frequency. Because of the increased amplitude the absolute responses to HT were diminished, but there was no clear evidence of specific antagonism (Fig. 1). The responses to HT and noradrenaline were depressed by the same amount. After benzoquinonium the dose of ACh had to be increased 10 to 50 times for the same response.

Mya arenaria

The threshold dose of HT was often between 0.5 and 2 μg./l., but often 10 or 100 times larger doses were needed. ACh inhibited the heart in concentrations of about 0.1 μg./l. and there were no large differences in the reaction of different animals as with HT. The reaction to carbachol was essentially the same as that to ACh, but the effective dose was 50 times greater. Adrenaline and noradrenaline stimulated, and isoprenaline inhibited, the heart in concentrations of 1,000 μg./l. or higher.

Both ephedrine and cocaine (100 mg./l.) increased the amplitude; in smaller concentrations (0.01–10 mg./l.) they had no action on the response to HT, but sometimes caused a partial block of the effect of adrenaline. Isoprenaline in smaller than threshold doses did not change the responses to HT or adrenaline.

The response to ACh was not definitely changed by physostigmine (10 mg./l.) or atropine (10 mg./l.). Tubocurarine (10–100 mg./l.) depressed it, so that 2–4 times the pretreatment dose was needed to have the same response. The inhibitory effect of isoprenaline was also prevented, but the response to HT remained unchanged.

Hexamethonium and decamethonium in concentrations up to 100 and 10 mg./l. respectively had no effect on the response to ACh or HT.

Histamine stimulated the heart in concentrations of 4 mg./l. or higher. Mepyramine did not prevent this action of histamine, but in concentrations of about 10 mg./l.

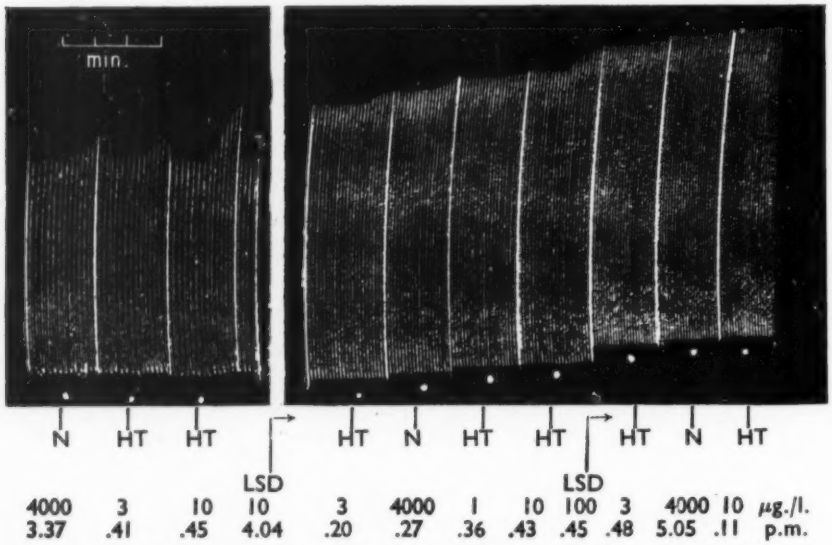

FIG. 1.—Heart of *Cyprina islandica*. 5 ml. bath. Concentrations μg./l. N, noradrenaline; HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide. N and HT in bath for 1 min. LSD in bath continuously.

it had a positive inotropic action, which appeared slowly in 5 min. or more.

Ouabain even in concentrations of 1 g./l. seemed to be without effect. LSD, like other derivatives of ergot, stimulated the heart, but had no specific effect on the response to HT.

The heart was quite sensitive to potassium ; an increase of the concentration of KCl from the ordinary 0.9 mg./ml. to 1.15 mg./ml. increased the force and decreased the frequency of the beat. Higher concentrations of KCl decreased the force and finally (2.15 mg./ml.) stopped the heart in diastole, which was followed by slow contraction to systole. The reaction to $CaCl_2$ was usually about the same as to KCl, but the concentration had to be increased in a ratio of 2 or more. The effect of a small increase of KCl could not be prevented by increasing the concentration of $CaCl_2$. Decreasing the concentration of KCl had the opposite actions—decrease in the force and increase in the rate. A decrease of $CaCl_2$ showed the same effect especially clearly. There was no clear change when the concentration of Mg was increased or decreased by 60%.

Cardium edule (*Cockle*)

The heart often beat with a constant amplitude and regular rhythm (8–15/min.), especially on the second day after the dissection. The response to HT was not usually maximal in 2 min., but even when the drug was only left in the bath for 1 min. the threshold concentration was 0.1–1 μg./l. LSD in concentrations of 1–10 μg./l. (left for 1–2 hr.) decreased the amplitude, and only when the heart was already nearly stopped was clear depression of the action of HT seen. 5-Benzyloxygramine and 6-methylgramine in concentrations of 1–10 mg./l. had the same action as LSD, but were 1,000 times less active. The action of these compounds was more or less completely washed off in 2–3 hr. Irregular or inert hearts were not stimulated by ergot alkaloids.

ACh was active in about the same threshold doses as HT. Benzoquinonium in concentrations from 1 to 10 mg./l. blocked about 1 to 100 threshold doses of ACh without having any effect on the response to HT. Atropine (1–10 mg./l.) did not block the response to ACh, and physostigmine (10 mg./l.) had a very weak sensitizing action, if any.

Adrenaline and noradrenaline (100 μg./l. or more) inhibited the heart like ACh (Fig. 2). In the presence of LSD (1–10 μg./l.) 10 times as much adrenaline and noradrenaline or more was needed for a given effect. This blocking effect was only partially abolished after washing until the following day. The action of the adrenaline was not clearly prevented by dibenamine (0.5–6.0 mg./l.) or by the gramines in the above-mentioned doses. Histamine was without effect in concentrations up to 5 mg./l.

Helix aspersa (*Garden Snail*)

The heart was stimulated by HT (*ca.* 1 μg./l.). The maximum reaction was reached in about 20 sec. and graded responses were obtained. LSD, ergometrine, dihydroergotamine, and hydergine all increased the amplitude and could not be shown to block HT. The above-mentioned gramines (1–10 mg./l.) may have had a weak anti-HT action, but they themselves depressed the rate and made the heart-beat irregular.

Adrenaline and histamine (1 mg./l.) were without effect, but ACh inhibited the heart in about the same doses as HT stimulated it. This reaction was not altered by physostigmine, atropine, benzoquinonium, decamethonium, hexamethonium or dibenamine in a concentration of 10 mg./l.

The heart sometimes beat well when superfused and this technique may be useful when increased sensitivity is needed.

Helix pomatia (*Edible Snail*)

The heart beat satisfactorily and reacted like *Helix aspersa* to HT and ACh, the threshold doses being 1 and 10–100 μg./l. respectively. Here again benzoquinonium (10 mg./l.) did not block the response to ACh, and LSD (1–100 μg./l.) and 6-methylgramine (1–50 mg./l.) were not HT-antagonists. Histamine, adrenaline and noradrenaline in concentrations of 5 mg./l. were without an effect.

Spisula (Mactra) solida

This heart started to beat in 5–60 min. after dissection, and the rate was 12–25/min. HT increased the amplitude in concentrations of 0.1–0.5 μg./l. Maximal response was reached within 45 to 60 sec. and relaxation was rapid. Tachyphylaxis was never found. Graded responses were obtained within large dose limits and for the maximum effect the concentration of HT was 1,000 μg./l. (Fig. 3).

LSD (1–10 μg./l.) increased the amplitude and rate and this obscured the action of HT. Ergometrine, hydergine, and dihydroergotamine had the same stimulant action. As Fig. 4 shows, the effect of a brief application of ergometrine was more or less permanent. 5-Benzyloxygramine and 6-methylgramine (Gaddum, Hameed, Hathway, and Stephens, 1955) were tried in doses from 0.1 to 10.0 mg./l. Usually the higher doses made

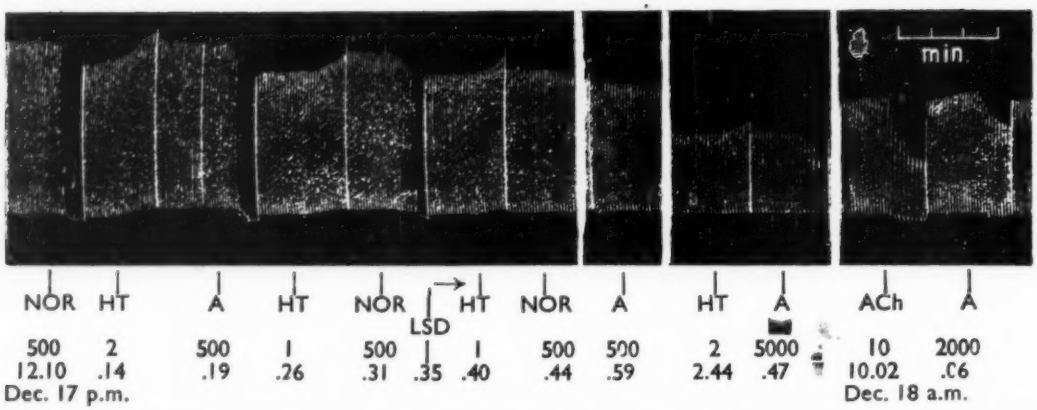

FIG. 2.—Heart of *Cardium edule*. 2 ml. bath. Doses ng. in bath for 1 min. NOR, noradrenaline; A, adrenaline; HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide. 12.35–2.48 LSD 1 μg./l.; 2.48–3.15 LSD 10 μg./l.

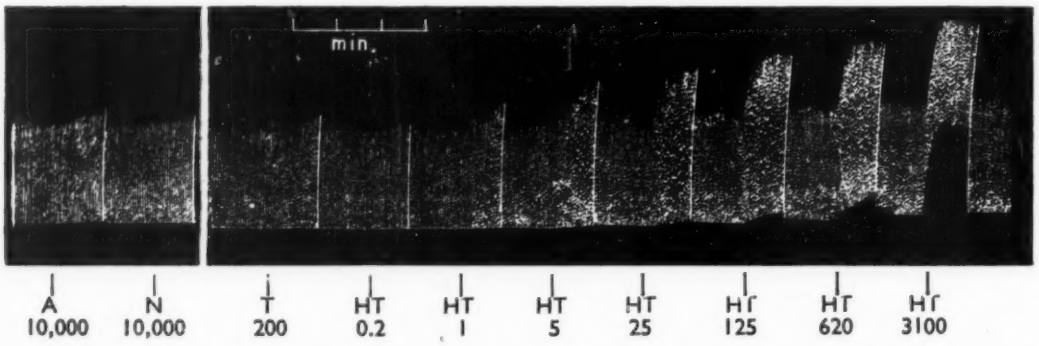

FIG. 3.—Heart of *Spisula solida*. Doses ng. in 2 ml. bath for 1 min. A, adrenaline; N, noradrenaline; T, tryptamine; HT, 5-hydroxytryptamine.

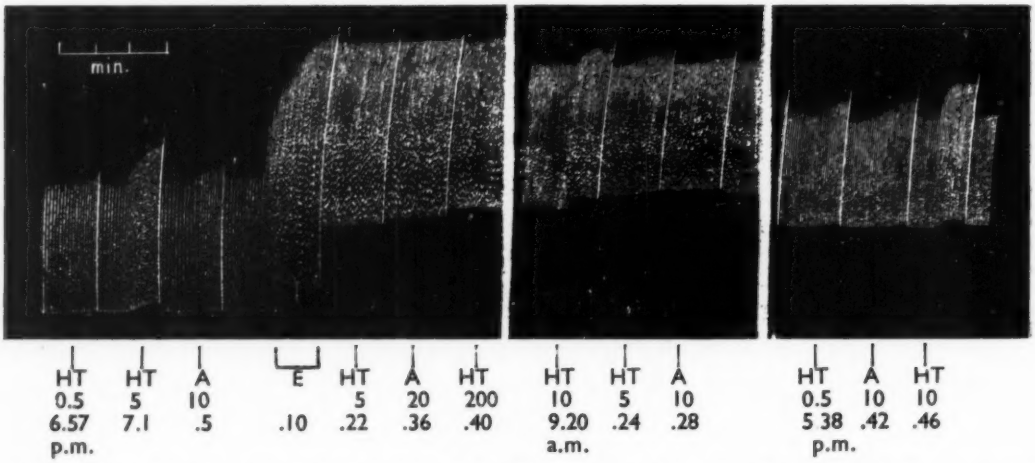

FIG. 4.—Heart of *Spisula solida* in 2 ml. bath. HT, 5-hydroxytryptamine (ng.) A, adrenaline (μg.); E, ergometrine (10 μg. for 90 sec.) increased the tone and rate for more than 24 hr. Responses to both drugs temporarily disappear.

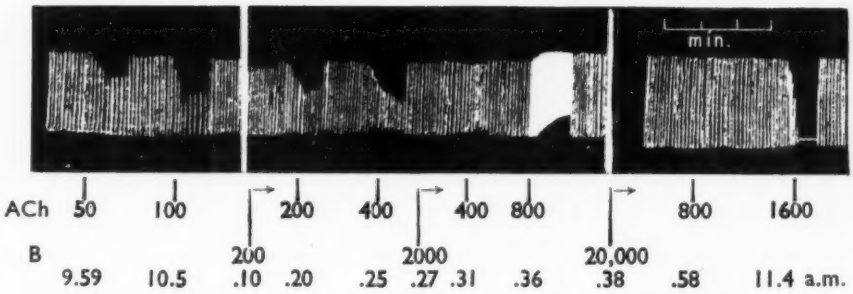

FIG. 5.—Heart of *Spisula solida* in 2 ml. bath. ACh, acetylcholine; B, benzoquinonium. Doses, ng. in bath.

the heart irregular, decreased the rate and increased the amplitude. There was some blocking activity, the highest gramine concentration being able to prevent the effect of 10–50 threshold doses of HT. This action, however, was not specific, the action of adrenaline being depressed as well. The prolonged application of HT itself causes specific densensitization of the guinea-pig ileum (Gaddum, 1953a), but similar experiments with the heart of *Spisula* showed no sign of desensitization.

ACh usually inhibited the heart in threshold concentrations of 1–100 μg./l. Sometimes large doses increased the rate and still larger doses (*ca.* 300 μg./l.) increased the force of the beat with increased tonus before the diastolic arrest. Carbachol had an inhibitory action in ten times higher doses. Physostigmine (10 mg./l.), atropine (10 mg./l.), hexamethonium (25 mg./l.), and decamethonium (25 mg./l.) had no action on the response to ACh. Tubocurarine (50 mg./l. or more) slightly diminished the response to ACh. The only substance found to be an efficient ACh antagonist on the heart of *Spisula* was benzoquinonium (Fig. 5) which is reported to have the same action on the heart of *Venus mercenaria* (Ludueña and Brown, 1952) and *Cyprina islandica* (Welsh, 1954). Benzoquinonium was more effective on hearts that were initially sensitive to ACh and relatively ineffective on resistant hearts. When it was present in concentrations of 1 and 10 mg./l. the threshold concentrations for ACh were about 50 and 300 μg./l. respectively. Adrenaline and noradrenaline stimulated the heart in concentrations of 1,000 μg./l. or more. Histamine in this dose was ineffective, and 0.5 U./ml. or more of pituitary posterior lobe hormones were needed to stimulate the heart. Adenosine, adenosine-5-phosphate, adenosine-diphosphate, and urea had a very small stimulant action in concentrations of 1 g./l. Substance P (P4, activity 18 U./mg., made from horse intestine) had no action in a concentration of 2 U./ml., and there was no reaction to bradykinin (20 μg./ml.) or to the uterus stimulating substance (4 mg./ml.) prepared from urine by Gomes (1955).

When NaH_2PO_4 was added in concentrations of 100–500 mg./l. or more, the amplitude was decreased sometimes after a brief increase.

The addition of 0.0004 N-HCl or more to the bath increased the amplitude, and 0.001 N-NaOH had a small inhibitory action.

Increasing the usual concentration of 0.65 mg./ml. of KCl to 0.9 mg./ml. caused a small increase of amplitude. This effect increased with the dose and at about 2 mg./ml. systolic arrest resulted. The effect of these changes in K concentration on the frequency was not constant.

An increase of the $CaCl_2$ concentration from the usual 1.1 mg./ml. to about 3 mg./ml. usually had a weak negative chronotropic effect, but the response to Ca, even in much higher doses, was not constant. A decrease of the concentration of Ca increased the frequency and decreased the amplitude, and the heart beat well in 0.5 mg./ml. of $CaCl_2$. Sometimes a decrease of Ca may help to make irregular hearts beat at a constant rate.

Experiments with Tissue Extracts

Tissues were extracted with acetone by the method of Amin *et al.* (1954), so that substances soluble in 95% acetone were extracted first, and the residue was extracted with dilute HCl.

The acetone-soluble fraction contains practically all the HT, and Amin *et al.* used it for the estimation of HT with rat uterus. They point out, however, that adrenaline may sometimes be present in sufficient amounts to interfere with the assay by inhibiting the uterus and so masking the effect of HT. Garven (1955) has found that this source of error can be largely eliminated by incubating extracts with the juice which may be pressed out of mushrooms, since this contains an enzyme which destroys adrenaline. In order to get further evidence of the meaning of results obtained in this way the HT-equivalents of acetone extracts of various tissues have been estimated (see Fig. 6) on *Spisula* heart and on the rat uterus, mushroom juice being used with the uterus, but not with the heart.

The results of these assays are shown in Table I. These two methods of estimation usually agreed fairly well with one another, but the values

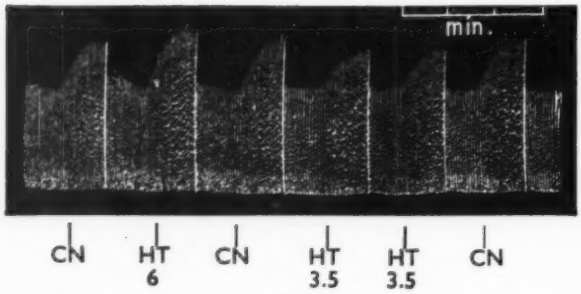

FIG. 6.—Heart of *Spisula solida*. 2 ml. bath. Drugs added every 3 min. and left in for 1 min. Assay of HT (doses in ng.). CN, acetone extract of dog's caudate nucleus. Each dose was equivalent to 21 mg. of tissue and contained less than 6 and more than 3.5 ng.

obtained for the HT-equivalent of extracts of the caudate nucleus with *Spisula* heart were about twice those obtained with rat uterus. Both sets of results are difficult to reconcile with those of Amin *et al.* (1954), who detected no HT in this tissue (<14 ng./g.). The properties of acetone-extracts of the caudate nucleus have therefore been studied in more detail.

TABLE I

THE ESTIMATION OF 5-HYDROXYTRYPTAMINE (NG./G.) ON THE HEART OF *SPISULA SOLIDA* AND RAT'S OESTROUS UTERUS

The values for the first three tissues were obtained in parallel assays. The tissues, except the last two, are from dogs.

	Spisula Heart		Rat Uterus	
Olfactory bulb ..	62 31 25	39	40 33 43	39
Caudate nucleus ..	88 200 60	116	44 117 24	62
Cerebellar cortex ..	12 7 8	9	<12 <20 <30	0
Sympathetic ganglia	<59 <19 <29	0	<28	0 (<28 bullocks)
Hypothalamus ..	224–526 (6 dogs)	370	220–330 (4 dogs)	280 (Amin *et al.*, 1954)
Human urine ..	100 89	95 ng./ml.		
Whole *Spisula solida*	706			

The estimates for the first three tissues on rat uterus were by Miss J. Garven.

The results suggest that the acetone-extracts of the caudate nucleus of dogs, cattle, and pigs did in fact contain HT, but that the results were complicated by the presence of other substances.

The solubility of the cardioactive substance in various liquids was similar to that of HT. When extracts were dried on the walls of evaporation flasks about 10% of the activity could be recovered with chloroform, 50% with 99% acetone, and 99% with 95% ethanol. When solutions of HT were treated in the same way similar results were obtained.

On ascending paper chromatograms with the upper alcoholic layer separating from a mixture of 4 vol. *n*-butanol, 1 vol. glacial acetic acid, and 5 vol. water (Partridge, 1948) a substance in extracts acting on both *Spisula* heart and rat uterus was found at the place corresponding to an R_F of 0.4. HT migrated in control strips at the same speed. In both cases the recovery was low (about 25%).

When solutions of HT containing N-NaOH were boiled for 5–10 min. all the activity was lost and similar results were obtained when extracts of dog's caudate nucleus were treated in the same way. The active substance in the caudate nuclei of cattle and pigs seemed to be much more stable and was unaffected by this treatment. This stability was, however, probably due to the presence of protective substances, since HT itself was also stable when added to these extracts. Extracts of the caudate nucleus of dogs did not have this protective action on added HT.

LSD is known to be an active and specific inhibitor of the effects of HT on rat uterus (Gaddum, 1953b). The immediate effects of extracts of the caudate nucleus were also abolished by LSD so that no response was seen in the first minute after the extracts were added to the bath, during which the main response to both the extract and HT had previously occurred. If, however, the extract was left in the bath, a large contraction of the uterus eventually occurred. These results were taken to indicate that the extracts contained HT, and another substance which caused a delayed contraction of the uterus after 1–1.5 min. This substance would not be expected to interfere with the use of rat uterus for the estimation of HT, provided the extracts were not allowed to act for more than 1 min. The main difficulty lies in the fact that estimates of the HT-equivalent of extracts of the caudate nucleus obtained with rat uterus were lower than those obtained with *Spisula* heart (Table I). Similar discrepancies were seen in experiments with acetone extracts of the caudate nuclei of cattle and pigs, in which the HT-equivalent estimated on the rat uterus was 20–80% of that estimated on *Spisula* heart. The results obtained with rat uterus may depend, however, on the doses used, since the log-dose-effect curve for extracts appeared to be flatter than the log-dose-effect curve for HT.

The most probable interpretation of these results is that the extracts contain substances which inhibit the response of the rat uterus to HT. One of these substances may be adrenaline, since Garven (1955) found an increase of the HT equivalent on rat uterus after treatment with mushroom juice, but this treatment was not as completely effective as would be expected if adrenaline were the only inhibitory substance present. If this is the correct explanation, then the assay on *Spisula* heart gives the best indication of the actual amount of HT present.

The dried residue, after extraction with 95% acetone, was then extracted with dilute HCl, as suggested by Amin *et al.* (1954). Such acid extracts of brain, liver, kidney, or urine stimulated the heart of *Spisula* when applied in a concentration corresponding to about 5 mg. of tissue or more in each ml. of bath fluid. This response started sooner and generally lasted longer than the response to HT and was mainly shown as an increase of the rate of the beat. These experiments were disturbed by vigorous foaming, but this was avoided when the extracts were made with 60% acetone. The substance responsible for the foaming then remained undissolved, but extracts still increased the rate of the heart of *Spisula*. The substance causing this effect was insoluble in petrol ether and stable at 100° in N-HCl or N-NaOH. It has not been identified with certainty, but it is possible that the observed effects were due to a mixture of potassium and phosphate.

Discussion

Real sea water is often used to suspend isolated tissues from marine molluscs. Artificial sea waters have been found more satisfactory and this appears to be mainly because the concentration of magnesium in real sea water is too high. This did not seem to matter in the early experiments in the autumn, but later on, in the winter and spring, the use of artificial solutions with a low magnesium content seemed to be more important.

In experiments with *Mya* or *Spisula* the usual effect of a decrease in the concentration of potassium, calcium or magnesium was a quicker and weaker beat and an increase of tone. An increase of the concentrations of these ions often had the opposite effects, but there were complications. For example, the final effect of an increase in the concentration of potassium was generally a slower and more forceful beat, but sometimes, in the autumn, it caused an initial quickening of the beat. We could find no evidence of an antagonism between potassium and calcium on the heart of *Mya* such as has been reported not only for the hearts of vertebrates but also for those of *Anodonta* (Hendrickx, 1945) and *Ostrea* (Jullien, 1931).

Most of these molluscan hearts were stimulated by HT in low concentrations, and by adrenaline or noradrenaline in concentrations about 1,000 times higher. The amounts of adrenaline or noradrenaline in most tissues would not be likely to interfere with the use of these hearts for the estimation of HT. Several of the species studied could be used for assays, but the best results were obtained with *Spisula solida*. The heart is rather small and some practice is required to set it up, but once it started beating it usually continued to give regular results for long periods and could sometimes be used for three days. The animals were usually easy to obtain and survived for reasonable periods in the laboratory. The effect of HT developed rapidly and disappeared rapidly when the solution was changed, so that the solutions to be tested could be applied at intervals of 2–4 min., except when very large doses were used. With low doses the time interval could be varied widely without affecting the result.

Some of the other species were not much studied because of the weakness of the muscle, or because it seemed to be difficult to obtain regular supplies of the animal, or because they did not survive long in captivity.

We have not found a really satisfactory specific antagonist for HT on molluscan heart. Welsh (1953) tried a few indoles, but they were excitatory themselves or ineffective. LSD, which is an effective antagonist for HT on rat uterus (Gaddum, 1953b), is said by Welsh (1954) to be also effective on the hearts of *Cyprina, Buccinum,* and *Venus.* In our experiments on the hearts of *Cyprina, Cardium,* and *Spisula* the antagonism has appeared to be unspecific, and feeble except when the heart was maximally stimulated by the LSD (Fig. 1). Other ergot alkaloids, including ergometrine, also stimulated the hearts of various molluscs and obscured the effects of HT and adrenaline (Fig. 4). Ergometrine has little or no action against HT on rat's uterus or perfused rabbit's ear. The stimulant action of these ergot alkaloids on the molluscan heart lasts for many hours after a brief application of the drug, and use has been made of this fact in assays of ACh on the hearts of *Venus* and *Mya* (Welsh and Taub, 1948 ; Hughes, 1955).

The inhibitory action of ACh on molluscan hearts forms the basis of well-known tests for this

substance. The heart of *Spisula* was often much less sensitive to ACh than those of certain other species and would probably not be especially suitable for the assay of this substance.

Physostigmine had no definite effect on the response to ACh and this confirmed the conclusion of most previous workers with other species (Bacq and Coppée, 1937 ; Welsh and Taub, 1948 ; Smith and Glick, 1939). All agree that atropine does not antagonize ACh on molluscan heart (Prosser, 1940) ; some say, however, that it paralyses the inhibitory nerves (Hendrickx, 1945). The only active antagonist of ACh is benzoquinonium. In *Venus mercenaria* this substance was effective in equimolar doses with ACh (Ludueña and Brown, 1952). In *Venus* Welsh (1953) found that benzoquinonium blocked the inhibition of the heart produced by stimulation of the visceral ganglion, and he mentions (Welsh, 1954) that this substance antagonizes ACh also on the isolated heart of *Cyprina islandica* and *Buccinum undatum*.

Benzoquinonium antagonized ACh in our experiments with *Spisula*, but high activity was only found when the hearts were initially very sensitive to ACh. The sensitivity of the heart in the presence of a given concentration of benzoquinonium appeared to be fairly constant, and, if the hearts were initially insensitive, the benzoquinonium had little effect. In some insensitive *Spisula* hearts there was only feeble blockage of the temporary stimulation and tonus increase after large doses of ACh even in a concentration of 10 mg./l. of benzoquinonium (Dose ratio = 3–4). The ACh receptors in pulmonates seem to be different from those in lamellibranchs. In *Helix aspersa* and *H. pomatia* benzoquinonium did not antagonize ACh nor did any other of the drugs tested. This fact limits the usefulness of *Helix* hearts for the assay of HT.

The heart of *Cardium edule* often gave regular results over long periods. HT and ACh in low concentrations had the same actions on this heart as they had on other hearts, but the actions of adrenaline, noradrenaline and the ergot alkaloids were unexpected. Adrenaline and noradrenaline caused inhibition even in concentrations which had no action on other species. The effect of LSD was also inhibition instead of stimulation, and was followed by a period in which the inhibitory effect of adrenaline and noradrenaline was absent (Fig. 2).

Histamine had no effect except in high doses, which sometimes stimulated or depressed the heart. Mepyramine did not antagonize the action of histamine on the heart of *Mya*, but itself stimulated this heart.

When HT and ACh are both present in a solution each may interfere with the estimation of the other. As Welsh (1954) has pointed out, it is therefore desirable to use antagonists to make the assay more specific. In experiments on HT, benzoquinonium may be used to exclude the effects of ACh, and in experiments on ACh LSD may be used to exclude the effects of HT. Since the effect of LSD may be feeble and unspecific it is particularly desirable in the latter case to determine by direct experiment how much HT may be present in the solutions to be tested without affecting the result.

Summary

1. The effects of various drugs on isolated hearts from a dozen species of molluscs have been studied.

2. The heart of *Spisula solida* provides a convenient method of estimating 5-hydroxytryptamine (HT) which generally stimulates it in a concentration of 0.1–1 μg./l. It is not sensitive to other known substances in tissue extracts, except acetylcholine, the effects of which can be abolished with benzoquinonium. The animal is hardy and its heart beats constantly for several days in isolation.

3. Estimates obtained in this way of the concentration of HT in some tissues agree with those obtained with rat uterus. A discrepancy was observed with extracts of the caudate nucleus and this is attributed to the presence of unknown pharmacologically active substances.

4. The heart of *Cardium edule* is inhibited by adrenaline.

5. Benzoquinonium antagonizes the effect of acetylcholine on the hearts of bivalves but not that on the hearts of snails.

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THE EXCRETION OF SALICYLATE

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Almost all administered salicylate is removed from the body by excretion in the urine, only very small amounts being eliminated in the faeces and sweat. The benzene ring of salicyl compounds is resistant to metabolic breakdown. Many drugs which are excreted in the urine are more toxic in renal failure, and smaller doses have to be prescribed—as, for example, streptomycin, hexamethonium, salts of potassium and magnesium, and ammonium chloride. This has not been described for salicylates, and it therefore seemed of value to investigate salicylate excretion both in normal subjects and in uraemic patients.

Sodium bicarbonate reduces the toxic effects of salicylate, and this was found by Smull, Wegria, and Leland (1944) to be associated with reduction of serum salicylate concentration. Smith, Gleason, Stoll, and Ogorzalek (1946) showed that this was due to increased rate of excretion of the drug in alkaline urine. A considerable but variable proportion of urinary salicylate is conjugated with glycine as salicyluric acid or with glycuronic acid. The raised excretion in alkaline urine is due to increased elimination of free salicylate (Parker, 1948). Further details of the fractionation of urinary salicyl compounds have been investigated by Alpen, Mandel, Rodwell, and Smith (1951) using salicylic acid labelled with ^{14}C at the carboxyl carbon atom. In man, 10–85% is excreted as free salicylate, 0–50% as salicyluric acid, and 15–40% as glycuronic acid conjugates. Gutman, Yü, and Sirota (1955), comparing the clearance of free salicylate with that of inulin, found the ratio C_{sal}/C_{in} to be considerably below unity in acid urines, but to be greater than one at urinary *p*H above 7.5. In this paper we have amplified previous work by studying salicylate excretion when the urine is made alkaline by hyperventilation and by ingestion of the carbonic anhydrase inhibitor, acetazoleamide. The effects of change in urinary volume produced both by water and by osmotic diuresis have also been studied. The theoretical and practical implications of the results obtained are discussed in detail.

METHODS

Observations were made on two healthy adult males (C. R. M. and M. D. M.), an adult female patient with normal renal function who was being treated with salicylates, and two adult males with chronic glomerulonephritis and uraemia. Precautions were taken to avoid any toxic reaction in the patients, but the normal subjects, who had more prolonged salicylate intake, suffered from tinnitus, high-tone deafness, and anorexia. Sodium salicylate was taken by mouth in a dose of 0.5 g. hourly, and clearance observations were made at stable salicylate levels of 15 to 18 mg./100 ml. In the normal subjects repeated observations were made during periods of acute change in urinary *p*H produced by taking sodium bicarbonate 10 g., acetazoleamide 250 mg., ammonium chloride 4 g., or by hyperventilation at 2.5 times the basal rate for 2 hr. In the patients a single period involving ingestion of acetazoleamide only was studied. The clearances in the uraemic patients were combined with determination of inulin clearance used as a routine renal function test. The effects of acute alterations in urinary volume were studied in the two normal subjects only. Water diuresis was obtained by rapid ingestion of 1 l. tap-water, and osmotic diuresis by rapid intravenous infusion of 300 ml. 25% mannitol.

The following analytical methods were used : serum salicylate, method of Smith *et al.* (1946), determination of the non-protein bound fraction of serum salicylate being made after ultra-filtration through cellophane ; fractionation and estimation of urinary salicyl compounds, method of Smith *et al.* (1946) ; chromatography of salicylate and salicyluric acid, method of Dalgleish (1955) ; serum and urinary inulin, method of Roe, Epstein, and Goldstein (1949) ; urinary *p*H, glass electrode ; urinary osmolarity, depression of freezing point using a Beckmann thermometer.

Statistical methods used in the analysis of the results were as described by Snedecor (1946).

RESULTS

Salicylate clearances were measured at reasonably stable serum levels of 15 to 18 mg./100 ml. The ultra-filterable fraction used for calculation of the free salicylate clearance varied from 22–44% of the total, with a mean value of 32%. At these

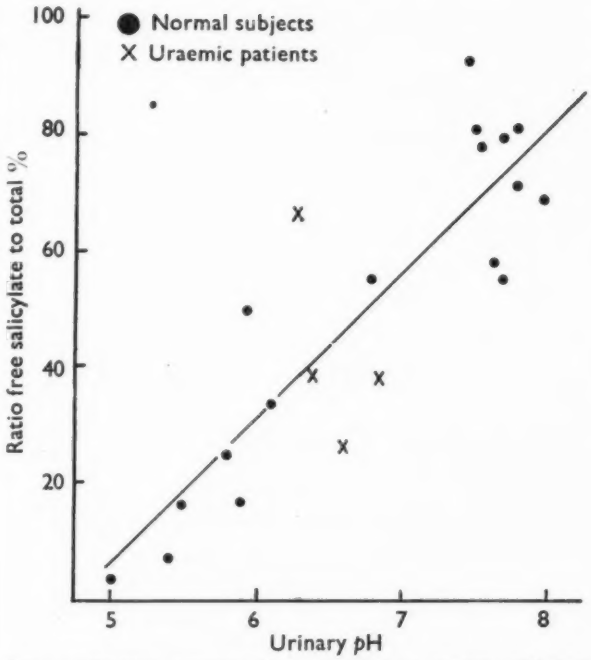

FIG. 1.—Relation between the percentage of urinary salicylate excreted as free salicylate and the urinary *p*H. The proportion of free salicylate in uraemia is within normal limits.

serum levels the excretion of combined salicylate ranged from 1.0–3.3 mg./min., with a mean value of 1.7 mg./min. There was no significant change in the excretion of combined salicylate with variation in urinary *p*H. In contrast, the excretion of free salicylate increased rapidly as the urine became more alkaline. Consequently, the free salicylate fraction was low when the urine was acid, and high when alkaline (Fig. 1). Both free and combined salicylate were excreted by the uraemic patients at low normal values, and in proportion similar to those in healthy subjects (Fig. 1). In particular, formation of salicyluric acid was not significantly reduced in either patient. This was confirmed both by chemical estimation and by chromatographic separation.

In Fig. 2, clearance values of free salicylate (C_{sal}) are plotted against urinary *p*H. The relation is obviously not linear; but Table I shows that the logarithmic regression is linear, so that log (C_{sal}) is directly proportional to urinary *p*H (correlation coefficient + 0.92). Three different agents—sodium bicarbonate, acetazoleamide, and hyperventilation—were used to make the urine alkaline, but the effect on the free salicylate clearance was identical with each stimulus. The results obtained with acetazoleamide, which produces a systemic acidosis and an alkaline urine, are of special importance, since they prove that urinary *p*H rather than systemic acid-base balance determines the free salicylate clearance. Ammonium chloride, producing a strongly acid urine, reduced the clearance almost to zero.

TABLE I

ANALYSIS OF VARIANCE OF RELATION OF LOG (C_{sal}) AND URINARY *p*H

*p*H values were grouped at intervals of 0·2 of a *p*H unit.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P
Within *p*H groups ..	14	15·61	1·11		
Logarithmic regression ..	1	14·91	14·91	372·8	
Deviation from regression	13	0·70	0·054		
Error	49	1·95	0·040	1·35	>0·20

Clearances in the uraemic patients were in the low normal range, and on two occasions exceeded the simultaneous inulin clearance which averaged 20 ml./min. in these patients. This may occur in normal people at urinary *p*H above 7.5 (Gutman *et al.*, 1955), but in the patients was seen at *p*H 7.3 and 7.35.

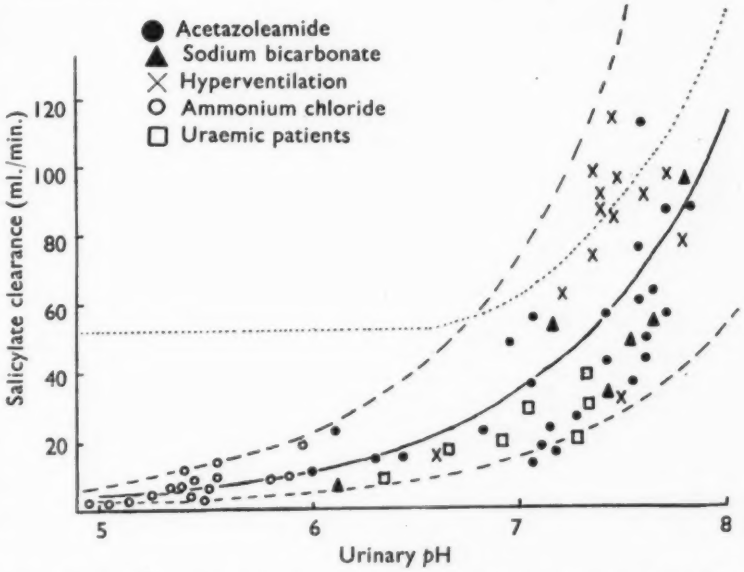

FIG. 2.—Relation between the free salicylate clearance and the urinary *p*H. The calculated regression line and the limits at twice the standard deviation are shown. The upper dotted line represents the average clearance calculated from total urinary salicylate. The log (C_{sal})/urinary *p*H relation remains unaffected whatever method is used to alter urinary *p*H.

The regression equation relating log (C_{sal}) and urinary *p*H was calculated to be:

$$\log (C_{sal}) = (0.52 \times pH) - 2.10$$

A similar calculation from the data of Gutman *et al.* (1955), in which sodium bicarbonate alone was used to alter urinary *p*H, gave the equation:

$$\log (C_{sal}) = (0.52 \times pH) - 1.92$$

The difference between the two results is not statistically significant. The regression coefficient of 0.52, which defines the rate of change of clearance with *p*H, will be shown later to be of great theoretical importance.

Fig. 2 gives the average values of the clearance calculated from total, as well as free, salicylate. This is of less theoretical but more practical importance, since it determines the actual rate of salicylate elimination from the body. There is no significant change in excretion of *p*H values below 7.0, but in alkaline urine the clearance rapidly increases with rising *p*H.

There is considerable scatter, largely due to variation in urine flow, in the (C_{sal})/urinary *p*H relation. Fig. 2 gives the limits at twice the standard deviation. Clearances obtained at low rates of urine flow were lower than the values calculated from the regression equation. Conversely, during water diuresis, higher values were found. Fig. 3 shows the relation between the deviation of the logarithms of observed and calculated clearance values and the urinary minute volume. As the urinary volume rises from 0.5 to 6.0 ml./min., there is a significant increase in clearance ($P<0.01$), but at still higher rates of flow no further rise occurs. This may explain the observation of Parker (1948) that dehydration tends to raise serum salicylate levels with consequent increased toxicity. If the urine is alkaline, however, variation due to flow is small compared with that due to *p*H change.

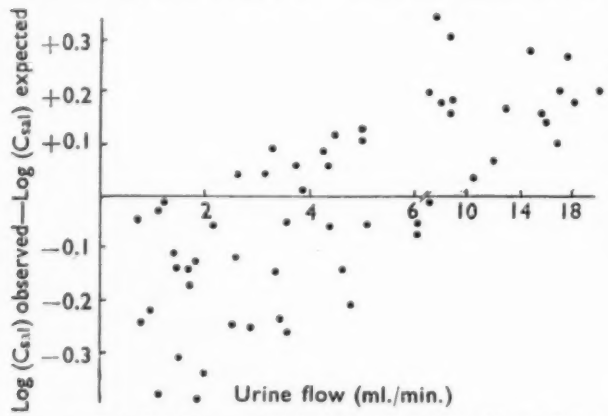

FIG. 3.—Relation between the deviation of the logarithms of the observed and calculated free salicylate clearance and the urinary minute volume. At low rates of urine flow values are less than the expected level and the reverse occurs during water diuresis. Note change of scale of abscissa at 6 ml./min.

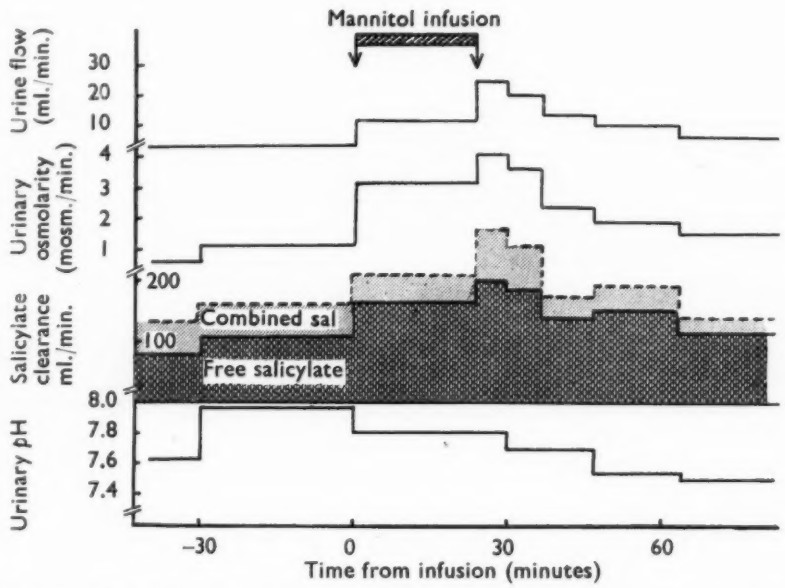

FIG. 4.—Effect of osmotic diuresis on the free salicylate clearance. The urine flow and the osmolar excretion increase proportionately more than the salicylate clearance.

Fig. 4 gives the values of clearances obtained during osmotic diuresis after rapid mannitol infusion. The urine was kept alkaline throughout by the ingestion of 15 g. sodium bicarbonate three hours previously. The urine volume rapidly increased from 3 to 24 ml./min., and the osmolarity from 1 to 4 mosm./min. Simultaneously, the free salicylate clearance rose sharply to twice the initial level. Osmotic diuresis therefore has a much greater effect on salicylate clearance than water diuresis. The theoretical implications of this will be discussed later.

DISCUSSION

The results show that the clearance of free salicylate is increased by alkalinization of the urine, whether this is produced by sodium bicarbonate, acetazoleamide, or hyperventilation. Thus, the main determinant of salicylate excretion is proved to be the reaction of the urine and not

systemic acid-base balance. Smith (1949) suggested that the explanation lay in selective tubular reabsorption of un-ionized salicylate molecules. This theory has been shown to be quantitatively inadmissible by Dalgaard-Mikkelsen (1951). The pK_a of salicylate, which is the *p*H at which there are equal numbers of ionized and un-ionized molecules, is as low as 3.0. In urine of *p*H 5.0 about 99% of salicylate is ionized, and the proportion increases with rising *p*H. The concentration of the un-ionized fraction is therefore invariably low, and selective tubular reabsorption would be quite inadequate to account for the large changes in salicylate excretion.

An alternative explanation put forward by Berliner (1954) is much more satisfactory, and, in a slightly modified form, is strongly supported by our data. Berliner states, "If relatively complete impermeability to the ionized form exists and if equilibrium between tubular lumen and peritubular fluid occurs, the concentration of un-ionized salicylate must approach equality within and without the tubule. Under these circumstances the observed changes in excretion (including excretion, in alkaline urine, of amounts in excess of those filtered) would be predicted and no active transport of salicylate *per se* would be required." We consider that this statement should be modified in that this equilibrium is in fact far from complete, and the important influence of variation in urinary flow must be considered.

The details of this theory are more easily appreciated by reference to the diagram (Fig. 5). Only the distal tubule need be considered, since this purely physico-chemical explanation excludes participation of the glomerulus and proximal tubule. A single distal tubule is represented by the tubular cells E surrounding the tubular lumen F. The tubule is bathed in peri-tubular fluid D, which approaches chemical equilibrium with an ultra-filtrate of the blood contained in the small arterioles and capillaries C. It must be assumed that the tubule is divided into two portions with widely different properties and functions, a more proximal portion A and a distal portion B. The former is concerned with adjustment of urinary *p*H by appropriate exchanges of hydrogen and ammonium ions for sodium in the tubular fluid according to the systemic acid-base balance. The cell membrane has selective permeability to salicylate molecules in that it is freely permeable to the un-ionized fraction, but does not allow passage of ionized salicylate. In contrast, part B of the tubule is concerned with selective reabsorption of water and is the final determinant of the rate of urinary flow. The cell membrane here is almost completely impermeable to salicylate molecules, whether ionized or un-ionized. The possibility that there is some overlap of function, or that there is a gradual and progressive change of property, is not precluded. There is histopathological evidence that in fact this latter portion of the nephron may lie in the collecting tubule rather than in the true distal tubule (Darmady, 1954).

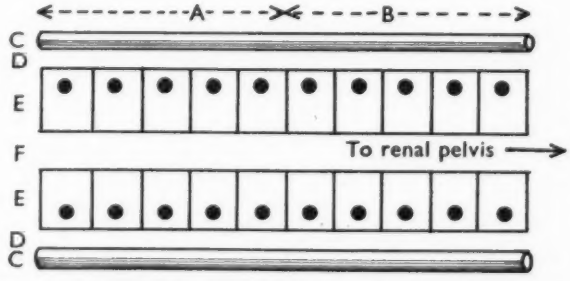

FIG. 5.—Diagrammatic representation of a length of distal tubule. In the more proximal portion, A, where urinary *p*H is determined, the tubule is permeable to un-ionized salicylate molecules but impermeable to the ionized fraction. In the more distal portion, B, which is concerned with determination of rate of urine flow, the tubule is almost completely impermeable both to ionized and to un-ionized fractions. Peri-tubular arterioles and capillaries are represented by C, peri-tubular fluid by D, tubule cells by E, and tubular lumen by F. Diffusion of un-ionized salicylate molecules from the peri-tubular fluid, D, to the tubular lumen, F, accounts for the marked influence of urinary *p*H on salicylate excretion.

Molecules of un-ionized salicylate diffuse freely from the peri-tubular fluid D into the distal tubular cells E, and finally into the fluid in the tubular lumen F. In any solution containing salicylate, the ratio of ionized and un-ionized salicylate molecules is determined by the Henderson-Hasselbalch equation:

$$pH - pK_a = \log \frac{[\text{ionized molecules}]}{[\text{un-ionized molecules}]}.$$

If the salicylate molecules diffuse into a highly alkaline urine a greater proportion become ionized, and therefore more salicylate must pass from the peri-tubular fluid to tubular lumen before equilibrium of the un-ionized fraction is reached. This would naturally result in a raised salicylate excretion in alkaline urine. The fact that urinary *p*H determines the rate of salicylate excretion rather than the final urinary salicylate concentration proves that the distal portion B of the nephron is almost completely impermeable to salicylate molecules. The slight influence of urine flow during water diuresis shows, however, that there is minimal diffusion even in this segment of the nephron.

The fraction $\frac{[\text{ionized molecules}]}{[\text{un-ionized molecules}]}$ can be shown to be directly proportional to the free

salicylate clearance. The concentration of the un-ionized fraction in serum and peri-tubular fluid must vary with the non-protein bound serum salicylate, since blood *p*H is almost constant. Similarly, the concentration of ionized molecules within part A of the tubule must be proportional to free salicylate excretion in unit time, since over 99% of this is ionized. Theoretically, then, the following relation should hold:

log (salicylate clearance) = urinary *p*H minus a constant.

Experimentally it was found that:

log (salicylate clearance) = (0.52 × urinary *p*H) minus a constant.

This highly significant discrepancy can be explained by the assumption that equilibrium of un-ionized salicylate between peri-tubular fluid and tubular lumen is not complete. In view of the limited time available for diffusion complete equilibrium is in fact most unlikely to occur. By Fick's law of diffusion, the rate of flow of a diffusing substance is proportional to its concentration gradient. This means that, at increased rates of salicylate excretion in alkaline urines, there is a higher concentration gradient of un-ionized salicylate between peri-tubular fluid and the tubular lumen, and consequently equilibrium is less complete. If full equilibration occurred, C_{sal} would be expected to increase tenfold for every unit rise of urinary *p*H, whereas in fact a tenfold increase occurs with every *two* units rise of *p*H.

Further confirmation of the diffusion theory is given by the results obtained during osmotic diuresis. Current views of renal physiology suggest that the volume of isosmotic fluid in the segment of the distal tubule A is proportional to the excreted osmolar load. Infusion of mannitol (Fig. 4) temporarily increased the urinary osmolar excretion fourfold. This would increase the volume of tubular fluid available for diffusion of salicylate by a similar amount. If equilibration were complete the salicylate clearance would be expected to rise correspondingly, but the observed increase was in fact half this—that is, twofold. Again, this can be explained by the assumption of a greater concentration gradient with increased rate of salicylate excretion.

The results in the uraemic patients show that excretion of salicylate may be almost normal despite gross reduction of the glomerular filtration rate. This is again to be expected on the diffusion theory, since glomerular function is not involved. Salicylate excretion in extreme renal failure may obviously be limited by gross reduction of the renal blood flow. The kidney cannot excrete a substance at a rate greater than that at which it is delivered by the blood. This aspect was not investigated, since accurate determination of renal blood flow in uraemia necessitates renal vein catheterization (Cargill, 1949).

Similar principles have been shown to govern the urinary excretion of ammonia (Clarke, Evans, MacIntyre, and Milne, 1955). Since ammonia is a basic substance increased excretion occurs in acid urines. The direction of the concentration gradient with ammonia is modified by its intracellular synthesis. The highest concentration is within the cell, and diffusion occurs both into the tubular lumen and outwards to the peri-tubular fluid and renal capillary blood. It is probable that a diffusion mechanism also determines the excretion of gentisic acid (Batterman and Sommer, 1953), and of the bases quinine (Haag, Larson, and Schwartz, 1943), nicotine (Haag and Larson, 1942), mepacrine, chloroquine, sontoquine (Jailer, Rosenfeld, and Shannon, 1947), and procaine (Terp, 1951). The observed data regarding the excretion of these substances are, however, not nearly so complete as those relating to ammonia and salicylate.

In summary, the diffusion theory explains almost all the observed facts of salicylate and ammonia excretion. One possible theoretical objection is that a purely physico-chemical mechanism is considered to perform osmotic work by concentration of either salicylate or ammonia. In fact, however, work is performed in the maintenance of a concentration gradient of hydrogen ion between blood and urine. In addition, it is probable that intracellular energy is necessary to maintain the peculiar selective permeability of the cell membrane to the un-ionized molecule.

The results are of practical pharmacological importance in the following respects:

(*a*) Therapeutic doses of salicylate can be prescribed in cases of uraemia of moderate severity without being excessively toxic. In severe renal failure, however, excretion may be impaired by gross reduction of the renal blood flow.

(*b*) In the treatment of salicylate poisoning the aim should be to produce and maintain a urine of maximal *p*H. It is not sufficient to make the urine alkaline to litmus. The reaction should be kept about *p*H 8.0. Acetazoleamide may prove to be useful, as it causes very rapid alkalinization of the urine, and produces a systemic acidosis which may counteract the respiratory alkalosis of salicylate intoxication. This drug, however, produces only temporary alkalinization of the urine,

and sodium bicarbonate is necessary to maintain a high urinary *p*H. The production of an osmotic diuresis by mannitol may also be useful in increasing salicylate excretion. Sufficient fluid should be given either orally or intravenously to prevent dehydration and maintain an adequate urine flow.

(*c*) Sodium bicarbonate should not be prescribed if salicylates are being given therapeutically. It is often considered that the prescription of a mixture of sodium bicarbonate and salicylate is exactly equivalent to the administration of a smaller dose of sodium salicylate alone. This is not correct. The aim in the therapeutic use of salicylates is to maintain a steady, non-toxic, but adequate blood level. This can be achieved only if the excretion of the drug remains constant. From the data in Fig. 2 it can be shown that log $(C_{sal}) = (0.52 \times \text{urinary } pH) - \text{a constant}$. Differentiating with respect to *p*H:

$$\log_e 10 \times \frac{I}{C_{sal}} \times \frac{d\,(C_{sal})}{d\,(pH)} = 0.52$$

$$\frac{d\,(C_{sal})}{d\,(pH)} = 0.23 \times (C_{sal})$$

Thus, the rate of change of salicylate excretion with respect to variation in urinary *p*H is proportional to the free salicylate clearance. Rapid variation of salicylate excretion therefore occurs when the urine is alkaline, with corresponding difficulty in the stabilization of serum levels.

Summary

1. The free salicylate clearance (C_{sal}) is related to urinary *p*H, log (C_{sal}) varying directly with *p*H.

2. The relation log (C_{sal})/urinary *p*H remains identical when urinary *p*H is altered by sodium bicarbonate, acetazoleamide, ammonium chloride, and hyperventilation. Thus, alteration in urinary *p*H rather than the systemic acid-base balance determines the rate of salicylate excretion.

3. Salicylate excretion in two patients with moderate uraemia was in the low normal range.

4. Water diuresis slightly increases the salicylate clearance, but osmotic diuresis has a much greater effect.

5. The observed facts are shown to be compatible with a theory that free salicylate is excreted by a process of tubular diffusion of un-ionized salicylate molecules rather than by the more familiar method of glomerular filtration and tubular reabsorption. Since the ratio of ionized to un-ionized salicylate increases with rising *p*H, this explains the more rapid excretion in alkaline urine.

6. The practical applications of the results in salicylate therapy and in the treatment of salicylate poisoning are discussed.

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THE REVERSAL BY OXIMES OF NEUROMUSCULAR BLOCK PRODUCED BY ANTICHOLINESTERASES

BY

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The reactivation by hydroxamic acids ($R—\overset{\overset{O}{\|}}{C}—NHOH$) of acetylcholinesterase (ChE) inhibited by organo-phosphorus anticholinesterases has been described by Wilson and Meislich (1953), Wilson and Ginsburg (1955) and Jandorf, Crowell, and Levin (1955). It has recently been found that the oximes ($\begin{matrix}R\\R'\end{matrix}\!\!>\!C=NOH$) are more potent reactivators of inhibited ChE than the hydroxamic acids and, like the latter, also react with the inhibitor (Childs, Davies, Green, and Rutland, 1955).

Most work on reactivators of inhibited ChE has been done with isolated enzyme preparations, and little on organized mammalian systems *in vitro* or on the whole animal. Roy and Kuperman (1955) have described reversal of the actions of TEPP by nicotinhydroxamic acid methiodide in isolated rabbit auricles and in strips of rabbit duodenum.

In the experiments described below, oximes have been tested for their ability to reverse the actions of organo-phosphorus anticholinesterase compounds at the neuromuscular junction in three preparations—the isolated rat phrenic nerve-diaphragm preparation, the rat gracilis muscle with electrical recording of muscle activity, and the cat tibialis anterior muscle prepared for close-arterial injection.

METHODS

Phrenic nerve-diaphragm preparations were obtained from 200 g. rats as described by Bülbring (1946). The nerve was stimulated with supramaximal square-wave pulses of 12.5 μsec. duration. Rates of stimulation varying from 12/min. to 200/sec. were used, so that both potentiation of response to single stimuli and Wedensky block of the higher rates of stimulation could be seen after addition of the ChE inhibitor.

For the experiments on the rat gracilis muscle, rats weighing between 250 and 400 g. were anaesthetized with urethane (0.5 ml. of 25% sol./100 g. intraperitoneally) and, after insertion of a tracheal cannula and an external jugular venous cannula, the right gracilis was prepared as described by Jarcho, Eyzaguirre, Talbot, and Lilienthal (1950). The obturator nerve twig, cut centrally, was stimulated with single shocks of 0.5 msec. duration from a "square-wave" stimulator at a rate of 10/min. and the voltage adjusted to supramaximal. The action potentials of the gracilis were recorded by means of one pair of fine platinum wire electrodes whose tips were beaded and adjusted to 2 mm. separation. The electrodes were held in a manipulator so that they could be traversed along the surface of the muscle in parallel with the muscle fibres. Action potentials were amplified by a capacity coupled amplifier and fed to one beam of a double-beam cathode ray tube, the other beam being used for msec. time marks. Single sweeps were recorded photographically on 35 mm. film, which was enlarged about 10 times and intervals measured to the nearest mm. One msec. was usually equivalent to 24 mm.

Cats were anaesthetized with pentobarbitone (32 mg./kg. intraperitoneally) and a tibialis anterior muscle was prepared for isometric tension recording and close-arterial injection of drugs as described by Brown (1938). The peroneal nerve was stimulated with single square-wave pulses of 0.5 mseç. duration at supramaximal voltage, and the response of the muscle to tetanic stimulation of the nerve at 50/sec. was also tested periodically.

In the experiments *in vivo* on rats and cats the animals were atropinized (1 mg./kg. i.v.) and artificially ventilated throughout the experiment to prevent the lethal muscarinic and central actions of the anticholinesterases. The inhibitors used were tetraethyl pyrophosphate (TEPP), di*iso*propyl phosphorofluoridate (DFP) and *iso*propyl methylphosphonofluoridate (sarin). Di*iso*nitrosoacetone (DINA) and mono*iso*nitrosoacetone (MINA) were the oximes generally used, although a few experiments have been done using tri*iso*nitrosopropane (TINP) and pyridine-2-aldoxime methiodide (PAM).

RESULTS

Rat Phrenic Nerve-Diaphragm Preparation

Addition of anticholinesterase drugs to the bath containing the preparation results in potentiation of the response to single shocks and block to

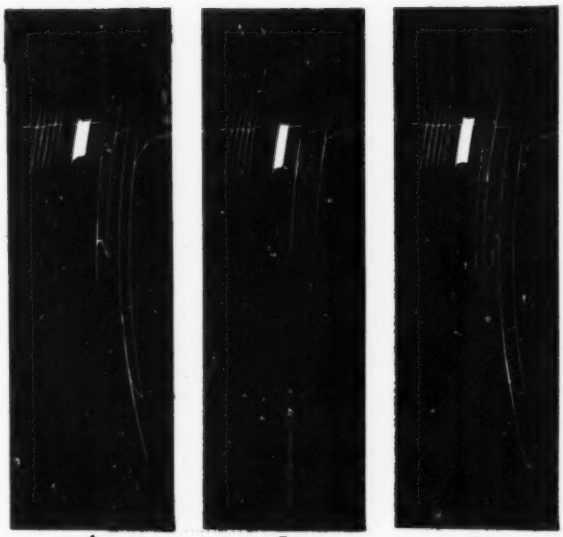

FIG. 1.—Contractions of isolated rat diaphragm to indirect stimulation at 0·5, 5, 50 and 100/sec. A, Control. B, 25 min. after TEPP, 2×10^{-7}M. C, 3 min. after MINA, 10^{-2}M. The preparation was not washed between records.

tetanic stimulation of the nerve (Wedensky block) —where the muscle responds only to the first of a train of stimuli applied to the nerve (Evans, 1951). The general conduct of an experiment was as follows. After the preparation had been set up and left for approximately 30 min. to reach equilibrium, the anticholinesterase was added in a concentration sufficient to cause block of the higher rates of stimulation as shown by a subsequent test. Oxime was then added and the responses to the various rates of stimulation again tested at intervals, usually 2 min. after addition of the oxime, and then at 5 min. intervals. A complete series is shown in Fig. 1, where reversal by the oxime of block at the higher rates of stimulation is seen. The results for a number of anticholinesterases and for the oximes used are given in Table I.

In the experiments in which the response to tetanic stimulation returned to normal, the preparations could be washed and the process of block by anticholinesterase, and of reversal by oxime, repeated with the same doses, and similar recovery times obtained.

Most of the experiments described above were done with short periods of contact between the anticholinesterase and the preparation (10 to 20 min.), but other experiments showed that duration of contact was not important in the reversal by oxime. In one experiment the preparation was left in contact with TEPP 2×10^{-7}M for 5 hr. before addition of oxime (MINA, 10^{-2}M) when complete recovery occurred in 3 min. Similarly with sarin (3.57×10^{-7}M), neuromuscular conduction returned to normal in 4 min. when MINA (10^{-2}M) was added 6 hr. after the anticholinesterase.

That the oximes act at the motor end-plates after addition of anticholinesterase has been demonstrated directly by recording the end-plate potential in a strip of isolated curarized rat diaphragm with the nerve attached as described by Jeffries (1953). The end-plate potential was markedly increased in size and duration by anticholinesterase (TEPP 10^{-7}M), but addition of oxime (DINA 10^{-2}M) to the bath resulted in a prompt return to normal.

One experiment has been done on the isolated phrenic nerve-diaphragm preparation of a kitten (197 g.). Block induced by sarin 3.57×10^{-7}M was reversed in 8 min. by the addition of DINA 10^{-2}M.

To test the direct action of oximes on muscle fibres rat phrenic nerve-diaphragm preparations were set up and oximes added to the bath without previous addition of inhibitor. The muscle was stimulated indirectly at a slow rate (approximately 5/min.) and periodically tested for its response to indirect tetanic stimulation and to direct stimulation. With DINA (10^{-2}M) no response to single stimuli was obtained after 2 hr. and the tetanic response was slight. Direct muscle stimulation resulted in only a feeble response and no recovery occurred after washing. MINA (10^{-2}M) appeared much less toxic, causing only slight reduction in contractions to single stimuli after 4 hr. With TINP (10^{-3}M), the response to single stimuli was almost completely abolished after 3 hr., but at this time a well-maintained tetanus could be obtained.

TABLE I

REVERSAL BY OXIMES OF NEUROMUSCULAR BLOCK PRODUCED BY ANTICHOLINESTERASES

Blocking Agent	Oxime	Time (min.) Necessary for Complete Recovery of Response to Stimulation at 100/sec.	
TEPP 2×10^{-7}M	DINA 10^{-2}M	5	
,, ,,	,, 10^{-3}M	20	
,, ,,	,, 10^{-4}M	—	Slight recovery in 30 min.
,,	TINP 10^{-3}M	—	No recovery in 90 min.
	then DINA 10^{-2}M	15	
,, ,,	MINA 10^{-2}M	3	
,, ,,	,, 10^{-3}M	7	
,, ,,	,, 10^{-4}M	—	Slight recovery in 65 min.
DFP 10^{-6}M	,, 10^{-2}M	2	
,, 10^{-5}M	,, ,,	45	30% recovery in 1 min., 50% in 3 min.
Sarin 3.57×10^{-7}M	DINA 10^{-2}M	2	
,, ,,	,, 10^{-3}M	15	
,, ,,	MINA 10^{-2}M	4	

As with DINA, these effects of MINA and TINP were not reversed by washing and appear to be a direct toxic action on the muscle fibres.

Pyridine-2-aldoxime Methiodide

This compound is particularly interesting, since it is the most potent *in vitro* reactivator of inhibited ChE so far obtained. The second order rate constants for reactivation of TEPP-inhibited red-cell ChE by MINA and PAM are 6.7 and approximately 500 respectively (Davies, personal communication). For sarin, the figures are 22.2 and approximately 200. Early experiments in which this oxime was tested on the rat diaphragm were disappointing in that recovery from anticholinesterase block was not obtained even with 10^{-2}M oxime, but the reason for this was later discovered to be a neuromuscular blocking action of the oxime itself. PAM does inhibit red-cell ChE to a certain extent particularly at the higher concentrations (45% inhibition at 10^{-2}M), but at 10^{-3}M, when neuromuscular block still occurs, its anticholinesterase activity causes only 4% inhibition. This neuromuscular blocking action of PAM is, however, reversed by washing the preparation, when recovery occurs immediately. In later experiments, therefore, the reversal of anticholinesterase block in the rat phrenic nerve-diaphragm by PAM was demonstrated by washing after the oxime had been added and left in contact with the preparation for a short time.

With 10^{-2} and 10^{-3}M-PAM, complete recovery from block by 3.57×10^{-7}M-sarin occurred after 3½ min. contact between the oxime and the preparation, which was then washed and tested. 5×10^{-4}M-PAM when added after block by sarin 3.57×10^{-7}M resulted in only partial recovery as shown by a test after washing. Further oxime was added to the same concentration, and conduction was restored to normal in 3½ min. At this low concentration the neuromuscular blocking action of the oxime seems to be insignificant, since after recovery there was no difference in response before and after washing.

In order to relate the reversal by oximes of anticholinesterase block to reactivation of the inhibited ChE, four experiments were done in which the ChE activity of the hemi-diaphragm used in the Bülbring preparation was estimated by the Warburg technique, after block by TEPP (2×10^{-7}M) and reversal by MINA (10^{-2}M). The remaining half of the diaphragm was divided into two, one part being used to obtain the normal ChE activity, while the other part was placed in normal saline containing TEPP (2×10^{-7}M) to provide a figure for the ChE activity after inhibition. The results are given in Table II.

TABLE II
ChE ACTIVITIES OF RAT DIAPHRAGMS
(µl. CO_2/30 MIN./100 MG.)

Expt.	Controls	Treated with TEPP	Treated with TEPP and MINA
1	50·5	(Not measured)	19·2 (38%)
2	56	11 (19%)	36 (65%)
3	61	11 (18%)	23 (38%)
4	57	19 (33%)	32 (56%)

Rat Gracilis

The gracilis contains two discrete motor end-plate zones, one close to the insertion of the muscle at the knee and the other nearer to the pelvic attachment of the muscle. With a pair of recording electrodes on the surface of the muscle between the two end-plate zones, two muscle spikes are obtained after stimulation of the nerve, originating in each zone and of opposite sign. If the electrodes are placed very close to one end-plate zone (usually that nearer the pelvis) the time interval between the two muscle spikes will approximate to the time taken for a muscle action potential to travel between the two zones. The interval will deviate

TABLE III
EFFECT OF DFP ON CONDUCTION VELOCITY OF RAT GRACILIS

Rat	Interval Between Spikes (msec.) Control	Interval Between Spikes (msec.) Minimum after DFP	Differences (msec.)	% Decrease in Conduction Time
1	2·70	2·08	0·64	23·7
2	2·82	2·25	0·57	20·2
3	2·42	1·97	0·45	18·6
4	2·62	2·01	0·61	23·2
5	3·05	2·58	0·47	15·4
6	2·52	2·12	0·42	16·7
Means	2·69	2·16	0·53	19·6

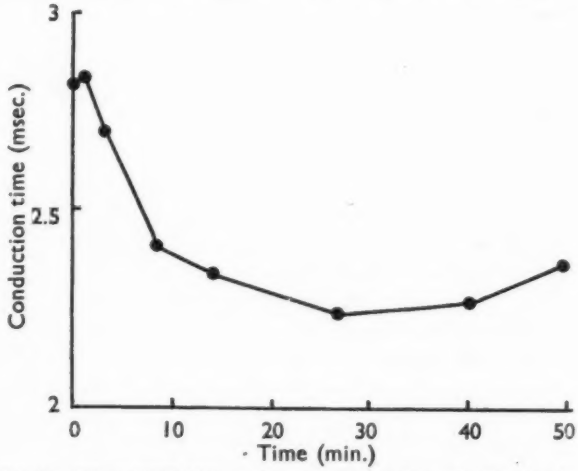

FIG. 2.—Effect of DFP (25 mg./kg. i.v.) on muscle conduction time in rat gracilis muscle. DFP given at zero time.

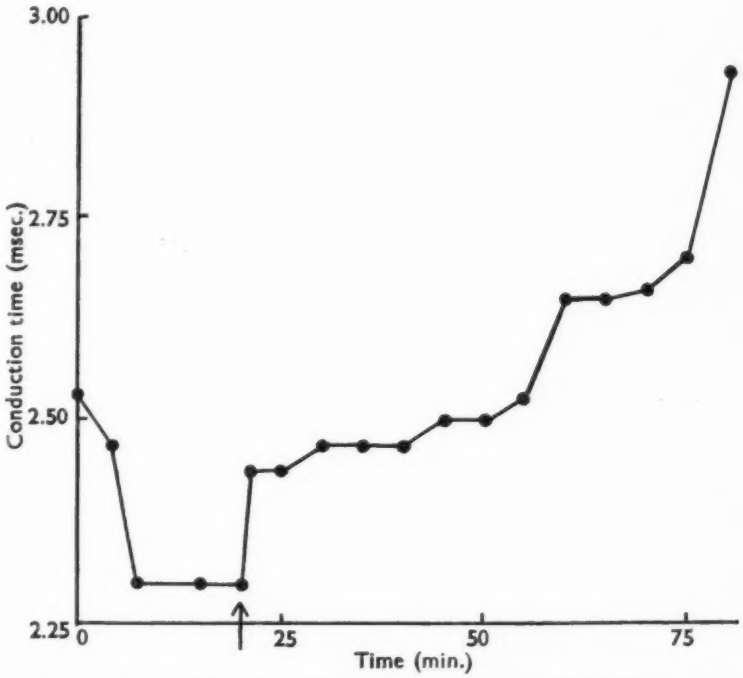

FIG. 3.—Conduction time in rat gracilis muscle. DFP (5 mg./kg. i.v.) given at zero time. MINA (50 mg./kg. i.v.) at arrow. Rat died at 80 min.

from this owing, firstly, to the additional nerve conduction time to the distal end-plate zone, and, secondly, to the muscle conduction time between the recording electrodes and the nearest end-plate zone. The positions of the end-plate zones can be found by traversing the electrodes across the muscle, when the crossing of an end-plate region is shown by reversal of the recorded action potential.

It has been found that DFP 2.5 mg./kg. i.v. causes a slowly developing reduction in the interval between the two spikes (conduction time), reaching a minimum between 10 and 30 min. after the injection. The results are given in detail in Table III, while Fig. 2 shows the time course of the change.

With a mean conduction distance of 11 mm. and the mean % difference of 19.6%, the injection of DFP 2.5 mg./kg. results in an apparent increase in conduction velocity from 4.1 to 5.1 m./sec. However this change is probably not due to a direct action of the DFP on the muscle fibre. Thus the occurrence of Wedensky block shows that the drug was acting at the motor end-plate, indirectly by potentiation of ACh. Single shock stimulation of the nerve would therefore cause a prolonged end-plate potential which would involve depolarization of the adjacent muscle membrane (Fatt and Katz, 1951), consequently reducing the effective conduction distance between the two end-plate zones. Muscular fasciculations started about 1 min. after the injection of DFP and continued throughout the experiment (up to 1 hr. duration). Repetitive firing occurred in the recorded muscle spikes in response to single shock stimulation of the nerve.

Injection of MINA 50 mg./kg. i.v. after the reduction in conduction time by DFP resulted not only in a rapid return towards normal but also in a further progressive increase in conduction time (Fig. 3). Experiments in which MINA alone was given showed that it had a direct effect on conduction time, causing a progressive increase in the interval reaching 10% above the control in 30 min. That there is, however, an action against the DFP-induced changes in addition to this direct effect acting in the same direction is shown by the initial rapid change in conduction time after the injection of oxime (Fig. 3) and also by the fact that repetitive firing due to DFP is promptly abolished (Fig. 4).

Cat Tibialis Anterior

It was found that neuromuscular block in the tibialis anterior due to sarin or TEPP could not be reversed by administration of oxime when both drugs were given by close-arterial injection. Thus block was produced by two injections of 2.5 μg. sarin followed by a further 5 μg., and was not relieved by injection of 250 μg. of DINA. When oxime was given close-arterially before sarin, it did not prevent the blocking action of the sarin. Since the oximes are readily washed out of isolated tissues (that is, before the prolonged slow toxic action occurs), it is concluded that,

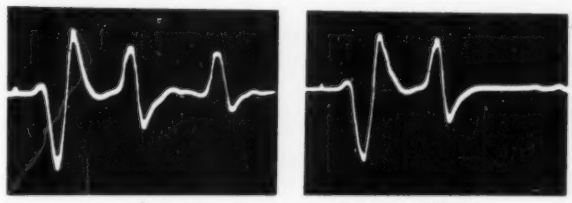

FIG. 4.—Rat gracilis muscle spikes to indirect stimulation. A, After DFP 5 mg./kg. i.v.; B, 1 min. after MINA 50 mg./kg. i.v.

after close-arterial injection, they are not "fixed" in the muscle, and thus a high local concentration of oxime would not be maintained for a sufficient time to obtain reversal of anticholinesterase block. Accordingly, in two experiments, oxime was given intravenously after neuromuscular block of the tibialis anterior had been induced by intravenous or close-arterial TEPP. In one experiment, 25 mg./kg. of MINA was given after 1 mg./kg. TEPP, both intravenously. The ability to maintain a tetanus returned fully after 25 min. Neuromuscular block was again produced by the injection of TEPP 1 mg./kg., and after the injection of 100 mg./kg. of MINA the recovery time was reduced to 11 min. In another experiment, TEPP (40 μg.) was given by close-arterial injection and produced only partial Wedensky block of the tibialis anterior. Injection of MINA 50 mg./kg. resulted in a maintained response to tetanic stimulation of the nerve after 8 min. In a control experiment in which only TEPP (1 mg./kg. i.v.) was given, the ability of the tibialis anterior to maintain a tetanus to indirect stimulation was only 21.5% of the pre-injection control after 60 min.

TABLE IV

EFFECT OF MINA (100 MG./KG. I.V.) ON CONTRACTIONS OF CAT TIBIALIS ANTERIOR TO INDIRECT STIMULATION AT 50/SEC. AFTER SARIN (300 μG./KG. I.V.)

Expt.	% Control Response after Sarin	Maximum % Control Response after MINA	Time (min.) from Injection of MINA to Maximum Recovery
1	1·5	82	20
2	0	100	17
3	1·0	98·5	20
4	0	88	34
5	0	72	45
6	0	91	26

A further series of experiments on cats was done with sarin, from which spontaneous recovery occurs more slowly than with TEPP, and also using a higher dose of oxime (100 mg./kg.). The results are given in Table IV. In two control experiments the recoveries which occurred from neuromuscular block by sarin (300 μg./kg. i.v.) were 2% in 60 min. from the injection of sarin in one experiment and 11% in 30 min. in the other.

DISCUSSION

The reversal by oxime of neuromuscular block due to anticholinesterase in the isolated rat phrenic nerve-diaphragm preparation is very rapid. With most of the anticholinesterases used, previous experiments have shown that prolonged washing is required to obtain recovery from block without addition of oxime. The time taken for recovery is related to the duration of contact of the anticholinesterase with the preparation; thus, for example, recovery after a 5 min. exposure to TEPP (2×10^{-7}M) occurs in 20 min., but with a 60 min. exposure to the same concentration 5 hr. washing is necessary for full recovery (Lovatt Evans, personal communication). However, experiments described above have shown that the time taken for reversal of block by oxime is the same whether the anticholinesterase has been in contact with the preparation for a few minutes or for many hours.

The *in vivo* experiments on the neuromuscular actions of the oximes have perhaps had less dramatic but nevertheless positive results. In the rat gracilis experiments, in addition to the change in conduction time and abolition of the additional spikes, it was noted that the generalized muscle fasciculations due to DFP poisoning disappeared after the injection of oxime.

In the experiments on cat muscle the results have been least encouraging in view of the time taken for recovery. Douglas and Matthews (1952) have shown that after 0.45 mg./kg. TEPP injected intravenously, the ability of the cat diaphragm to maintain a tetanus returns spontaneously to near normal in 50 min. With the tibialis anterior, which is blocked more rapidly after cholinesterase than muscles such as the soleus and diaphragm (similar to the action of decamethonium as described by Paton and Zaimis, 1951), it was found that MINA 25 mg./kg. i.v. caused a return of tetanic contraction in 25 min. after injection of 1 mg./kg. of TEPP. In a control experiment in which only TEPP (1 mg./kg. i.v.) was given, no recovery had occurred in 25 min. The series of experiments in which sarin was used also showed that injection of MINA resulted in a slow return of the ability of the tibialis anterior to maintain a tetanus. The slow rate of the recovery is thought to be due to the local concentration of the oxime being too low. That there is no species difference has been shown by the rapid reversal of sarin block by oxime in the isolated kitten diaphragm.

The mechanism of action of the oximes in reversing neuromuscular block appears to be entirely due to the reactivation of inhibited ChE. Thus in the experiments in which the ChE activities of phrenic nerve-diaphragm preparations were measured, the addition of oximes after TEPP caused an increase in ChE activity which accompanied the return of normal function. Furthermore, the oximes had no effect on block of phrenic nerve-diaphragm conduction produced by (+)-tubocurarine (1.1×10^{-6}M), succinylcholine

chloride (3×10^{-5}M) and decamethonium bromide (2×10^{-5}M). The direct toxic action of the oximes on muscle, however, seems to be an action on the fibre, since the response to direct stimulation is reduced (isolated rat diaphragm) and the conduction velocity is progressively slowed (rat gracilis *in vivo*).

The experiments described above have shown that the oximes have considerable potentiality against neuromuscular block from anticholinesterases. However, the required concentrations of oximes *in vitro* are too high and the *in vivo* dose necessary to reverse block from an anticholinesterase is close to the toxic dose. PAM, while very effective as an *in vitro* reactivator, suffers from the disadvantage of having neuromuscular blocking actions of its own.

Summary

1. The actions of oximes on neuromuscular block due to anticholinesterases have been studied.

2. Wedensky block in the isolated rat phrenic nerve-diaphragm preparation induced by TEPP, DFP or sarin was rapidly reversed by di*iso*nitrosoacetone and mono*iso*nitrosoacetone.

3. Pyridine-2-aldoxime methiodide reversed block due to anticholinesterases, but this could only be demonstrated after washing, since the oxime itself caused neuromuscular block at higher concentrations.

4. In the rat gracilis muscle *in vivo*, DFP caused an apparent increase in conduction velocity which rapidly returned to normal after injection of oxime.

5. Block in the cat tibialis anterior due to intravenous or close-arterial TEPP or intravenous sarin was slowly reversed by intravenous injection of oxime.

6. The oximes have a direct toxic action on muscle, causing a reduction of contraction height and a slowing of conduction velocity.

7. The reversal by oximes of neuromuscular block appears to be due to reactivation of ChE. Block by (+)-tubocurarine, succinylcholine or decamethonium was unaffected.

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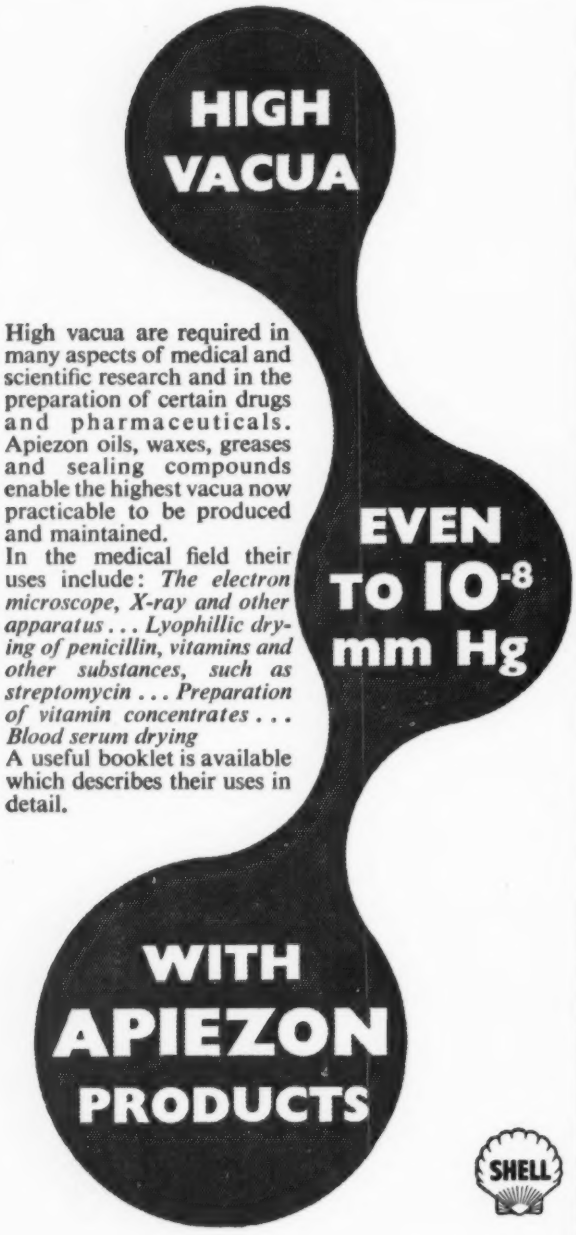

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